ELECTROPHORETIC PROFILE OF THE GENERAL PROTEINS IN THE GREEN (*Perna viridis* Linnaeus) AND THE BROWN (*Perna indica* Kuriakose & Nair) MUSSELS

DISSERTATION SUBMTTED
IN PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
MASTER OF FISHERIES SCIENCE
IN MARICULTURE OF THE
CENTRAL INSTITUTE OF FISHERIES EDUCATION
(DEEMED UNIVERSITY)
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Dedicated to my

Parents

DECLARATION

I hereby declare that this dissertation entitled **"Electrophoretic Profile of the general proteins in the Green (*Perna viridis* Linnaeus) and the Brown (*Perna indica* Kuriakose & Nair) mussels"** has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar titles or recognition.

Kochi

July, 1998

ABINASH PADHI

CERTIFICATE

Certified that the dissertation entitled **"Electrophoretic profile of the general proteins in the Green (*Perna viridis* Linnaeus) and the brown (*Perna indica* Kuriakose & Nair) mussels"** is a bonafide record of the work carried out by Mr. Abinash Padhi under my guidance and supervision and that no part thereof has been presented for the award of any other degree, diploma or any other similar title.

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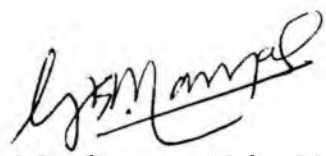

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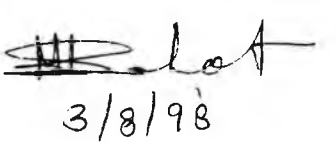

सारांश

हरा सँबु *पी. विरिडिस* और भुरा उँबु पी. *इंडिका* सौंबु वर्ग की दो जातियाँ है । इनकी एक संदेहित जाति दाझिण भारत मों पाई जाती है। इसकेलिए *पी. विरिडिस* और *पी. इंडिका* के प्रोटीन और *एनजाइम* प्रोफाइल (एम.डी. एच) पर अध्ययन चलाए थे । अध्ययन से प्यक्त हुआ कि दोनो जातियो के प्रोटीन और एनजाइम प्रोफाइल संयुक्त रूप से अलग अलग है । दोनो जातियो के विविध उतको मे निहित प्रोटीन प्रोफाइल अलग अलग जातियो के अभिलक्षण प्यक्त करने वाले थे । इसी प्रकार हरा सँबु और इसका संदेहित सँकर जाति का प्रोटीन प्रोफाइल एक समन था; वैसे भुरा सँब और इसका संदेहित सँकर जाति का भी । अध्ययन से यह स्पष्ट होता है कि संदेहित जातियाँ असल में अलग अलग जातियो नहीं बालिक उनके रँगरूपिम हैं ।

दोनों जातियों और इनके संदेहित सँकर जातियाँ

में चलाए आकारमापी अध्ययन भी उपयुक्त अनुमान को मजबुत करता है ।

ABSTRACT

The electrophoretic profiles of the general proteins and the selected enzymes (MDH and EST) were examined in the green mussel, *P. viridis* and the brown mussel *P. indica* and their suspected hybrids from South India. The protein and the enzyme profiles were distinctly different in the two species. The protein profiles in different tissues of the green and brown mussels were found tissue and species specific. The two species are genetically different. The protein and the enzyme profiles in the brown and suspected 'brown type' hybrids were similar and that of the green and the suspected 'green type' hybrids were also similar. The suspected hybrids may be the colour morphs of the respective species. Morphometric studies on these two species and the suspected hybrids also corroborated the above findings.

ACKNOWLEDGEMENTS

I express my deep sense of gratitude to Dr. M.K. George, Senior Scientist, C.M.F.R.I, Kochi and Major Advisor, for his constant help and in giving me the sumptuous guidance for structuring a proper plan of work, which infallibly assisted in completing my work in due time.

I am obliged to our adorable Director, Dr. M. Devaraj for providing me necessary facilities for my study.

I remain deeply indebted to Dr. C. Suseelan, Senior Scientist and Officer in-charge, PGPM, for his constant encouragement and co-operation during my course work.

I acknowledge my deep sense of gratitude to Shri T.S. Velaudhyan and Dr. (Mrs) Manpal Sridhar, Senior Scientists, members of Advisory committee, for their help and inspiration during my dissertation work.

I am rendering my sincere gratitude to Dr. P.C. Thomas, Senior Scientist, CMFRI, Dr. A. Gopal krishnan, Senior Scientist NBFGR Kochi unit, Dr. K.K. Appukutan, Senior Scientist, Mrs. V. Kripa, Scientist, CMFRI, Dr. P.S. Kuriakose, Principal Scientist, and OIC , Calicut Research Centre of CMFRI and Dr. G.P.K. Acharya, Senior Scientist, Vizhinjam Research centre of CMFRI for their help and encouragement.

Timely help rendered by Shri T.V. Sathianandan and Shri. K.N. Kurup, Scientists in statistical analysis are gratefully acknowledged.

My special thanks to Mr. P.M. Aboobekar, Technical officer, PGPM for his kind co-operation during my course work. I also extend my thanks to Mr. Andrew Mathew, Mr. K.T. Thomas, Vizhinjam and Mr. V.G. Surendranathan, Calicut, research centres of CMFRI, Technical officers and supporting staff Mr. P. Harshakumar for helping me during specimen collection. I am thankful to Mr. K. Raghavan for his help in taking photographs.

I remain deeply indebted to Miss. Bindhu Paul and Miss. V. Sapna, SRF's, without whom it would have not possible to complete my work within the stipulated time. Their constant help and encouragement made me to carryout this work..

I convey my sincere gratitude to Mr. M.M Shirdhenkar and Mr. V. Terrence Rebello, PhD scholars, for their constant help and valuable suggestion throughout my study period. I also thank Mr. M.P. Paulton, Technical Assistant for his timely help and to my friend Mr. S. Prasanth for his hearty co-operation and in giving me moral support during my work.

I wish to thank my batchmates especially Tushara, Bindhu, Raihanathu, Smitha, Krishna, Gajanan, Kannan, Rajesh and to my Junior Sandeep for their ready and untiring help.

I express my heartfelt thanks to my seniors, Binaya Bhai and Bhuyan Bhai for sending me the chemicals and references. I am also thankful to Ms. Jeeja Sudhir, Ram's Computech, Mamangalam for her help in desk top publishing of my dissertation.

I owe a lot to ICAR for awarding me Junior Research Fellowship during my study period.

Last but not least, I also thank all the authors and publishers whose books, Journals and articles were used for the preparation of this dissertation.

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1. Introduction

Mussels of the family Mytilidae are the commonest of marine molluscs. and constitute an important element in the ecology of coastal waters. Mussel meat is nutritious and good to eat. The mussel resources are exploited from their natural beds. Mussels are also cultured in many nations including India. As they are sessile filter feeders and have been shown to be effective concentrators of trace toxic substances, mussels are now being widely used as biomonitoring organisms for coastal water quality. They have been extensively used as model organisms in many scientific studies and a vast body of data is available from basic physiological, biochemical, genetic and toxicological investigations.(Gosling, 1992a)

Four hundred species of *Mytilus* and seventy one species of *Perna* are known to occur under the family Mytilidae. Since, many of these species have confusing common morphological characteristics, accurate classification and identification of mussel species is a practical problem to the taxonomists and the biologists (Siddall, 1980). For example, until recently, the mussels of India, popularly known as the green and brown mussels, were classified and described under the genus *Mytilus* where as these mussels were redescribed and put under the genus *Perna*. Thus the green mussel was described as *Perna viridis* where as the brown mussel was described and classified as a new species with the name, *Perna indica* (Kuriakose & Nair, 1976). A detailed account on the fishery and biology of the Indian green and brown mussels as well as their culture potential have been reported by CMFRI (CMFRI Bulletin 29) and during the national seminar on the shell resources and

farming in 1987 (CMFRI Bulletin 42, 1988). The green and brown mussels have distribution along the east and west coasts of India (Fig. 1).

For scientific exploitation, farming and conservation of any fishery resouses, an accurate identification of the species and its populations is essential (Allendorf *et al.*, 1987). The recent identification of the green and brown mussels of India was done on the basis of morphological phenotypes which can be influenced by the ecological and environmental parameters. However, gene controlled protein profiles of organisms are very valuable characteristics for accurate identification of the species and its populations, since protein / enzyme characteristics are much less influenced by the non -genetic parameters (Bye & Ponniah, 1983). Thus the biochemical genetic description of the species is more accurate and dependable. Besides, the detectable genetic differences can become complementary to the morphological differences already used for identification of the species. In this respect, the question arises that whether the morphologically described green mussel *P. viridis* and the brown mussel *P indica* are also genetically different species? Moreover , the mussel specimens with apparent mixed morphological characteristics were observed along with the green and brown mussels. These were suspected as varieties/hybrids of the green and brown mussels. These are two types. For practical purpose, one was labelled as 'brown type' hybrid as it had brown mussel shell shape but with green shell colour. The other 'green type' hybrid had green mussel shell shape but with brown/green shell colour. Are these specimens hybrids or varieties of the green and brown mussels? The aim of the

present study was to find out answers to the above questions. In the present study, morphometrics of these two species not considered by earlier workers (Kuriakose and Nair, 1976) were also used for obtaining additional informations on the morphological differences of the species. The dissertation contains the results of the present investigation on the protein profiles and morphometrics of the green and brown mussels of India as well as their suspected hybrids.

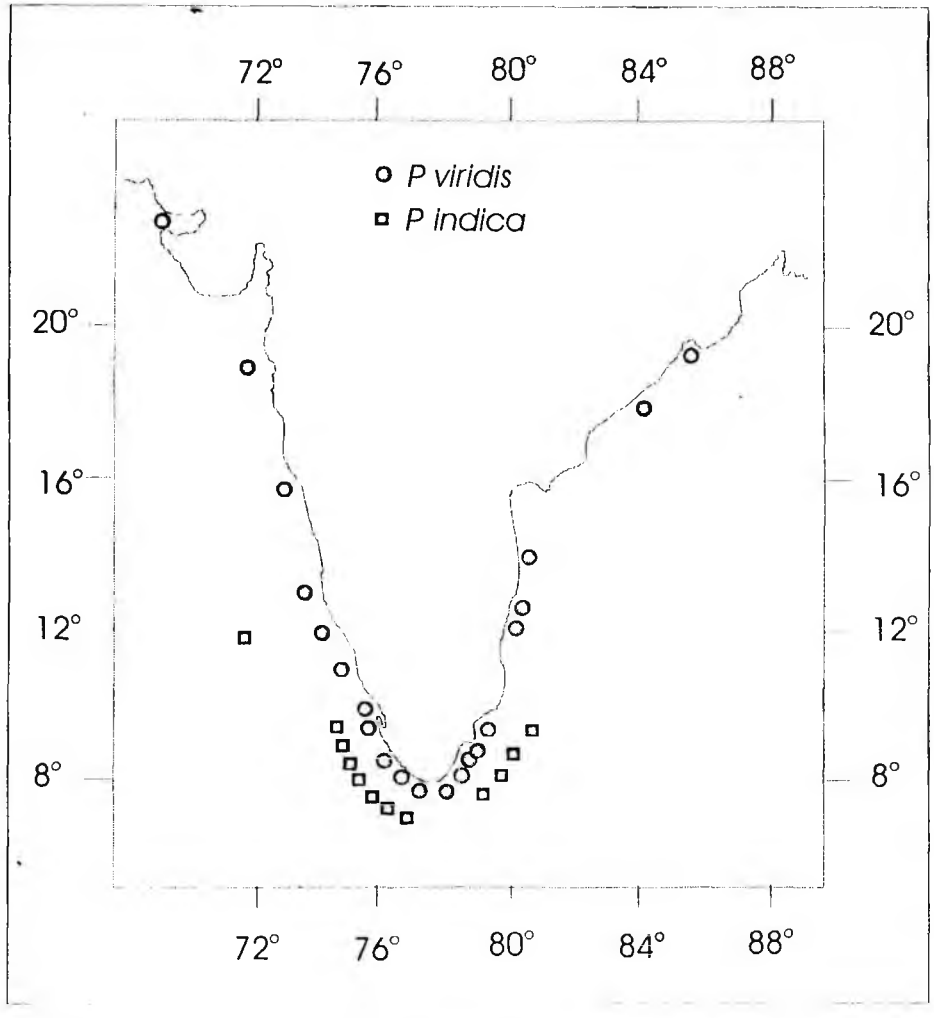


Fig.1. Distribution of *P viridis* and *P indica* along the Indian coast

2. Review of Literature

2.1 TAXONOMY

Invertebrates generally have higher level of genetic diversity than vertebrates as measured by protein electrophoresis (Nevo, 1978). The Mytilidae or true mussels, demonstrate a great deal of variation in morphological features which are taxonomically important in the Bivalvia . Thus the taxonomic status of species within the Mytilidae is often confused. For want of reliable morphological features by which to distinguish species and genera, the inconsistent or 'plastic' character of gross adult shell morphology has, in the past, shaped the family's hierarchy. The existence of physiological races and a wide range of eco-morphs in the Mytilidae has also complicated the interpretation of experimental evidence and created a taxonomic challenge for the researcher working with mytilids. (Siddall, 1980).

In 1932, the rosters of Index Animalium reported on the use of approximately 400 different species names in the genus *Mytilus* and another 71 names in the genus *Perna* (Siddall, 1980). Unfortunately, the name *Perna* had been used to describe two genera of mytilids, *Perna* (Retzius, 1788) and *Modiolus* (H and A.A Dams, 1858) and a genus of *Pteriacea, Isognomon* (Bruguiere, 1792). When Retzius (1788) set forth the genus *Perna*, he listed the type specimen as *Perna magellanica* which according to Lamy (1936-1937) is synonymous with Linnaeus *Mya perna* and whose holotype was presumably collected from the straits of Magellan. Both are properly referred to as *Perna perna* (Lamy 1936-37). *P magellanica* as described by Retzius must not be confused with *Mytilus*

magellanicus Chemnitz which, as Soot-Ryen (1955) details, probably belongs to the genus *Aulacomya.*

Linnaeus (1758) first described *M viridis* while Gmelin (1791) is credited for *M canaliculus.* The several works of Born, Chemnitz, Dillwyn, Gmelin and Lamarck expanded the list of species in the genus *Perna* but Hanley (1843, 1855) temporarily reversed this trend by lumping together several species. In Hanley's work (1843) the synonymy of *M viridis* L. and *M smaragdinus* Chemnitz was described as was the synonymy between *M canaliculus* Gmelin and *M latus* Chemnitz..

Von Ihering (1901, 1907) and Jukes-Browne (1905) discussed hinge and ligament structures and muscle scars as bases for establishing the taxonomic hierarchy of the Mytilidae. This work laid the foundation for Lamy's (1936-1937) analysis of museum specimens involving both the genera *Mytilus* and *Perna.* The confused taxonomic interrelationships involving *Mytilus* and *Perna,* which developed in the 19th century were simplified by Lamys comprehensive works followed by those of Soot- Ryen (1952, 1955). Dodge (1952) regarded *Chloromya* as a sub genus *Mytilus.* In 1952. Soot-Ryen divided the genus *Perna* into two groups (1) those having pitted resilial ridge belonging to the genus *Chloromya* and (2) having a compact resilial ridge forming a new genus *Choromytilus* with the genotype *C chorus* Molina 1782. Soot-Ryen (1955) showed *Chloromya* to be an invalid synonym for *Perna* and clarified much of the

taxonomic nomenclature of the mytilids by retaining *Perna* (Retzius, 1788) for those species of mytilids resembling *Mytilus* but which have a pitted resilial ridge and discontinuous posterior retractor muscle scars and lack an anterior adductor muscle. Dance (1974) placed *C chorus* Molina in the genus *Perna* but gave no new evidence supporting this change. The close taxonomic relationship among the genera *Mytilus*, *Perna*, *Choromytilus* and *Aulacomya* are briefly described by Soot-Ryen (1952). Siddall (1980) described several larval and adult characteristics of the three living species currently placed in the genus *Perna* (*P perna, P viridis and P canaliculus*)

Lamy (1936) recognized the following as distinct species of *Mytilus*: *Mytilus edulis* Linnaeus, 1758 from Northern temperate latitudes, *Mytilus galloprovincialis* Lamarck, 1819 from the Mediterranean sea, *Mytilus trossulus* Gould, 1850 from the pacific coast of North America, *Mytilus chilensis* Hupe, 1854 from Chile, *Mytilus platensis* Orbigny, 1846 from Argentina, *Mytilus planulatus* Lamarck, 1819 from Australia and *Mytilus desolationis* Lamy, 1936 from the Kerguelen Island. Soot-Ryen (1955) recognised. *Mytilus coruscus* Gould, 1861 (= *M crassitesta* Lischke, 1868) from Japan and China, and *Mytilus californianus* Conrad, 1837 from the pacific coast of North America, as distinct species, but considered most of the species described by Lamy to be subspecies of *M edulis*. Also, the Newzealand mussel *Mytilus aoteanus*, first described by Powell (1958), has since regarded as a subspecies of *M edulis* by Fleming (1959). More recently, Scarlato and Starobogatov (1979) have described what they

consider to be two new subspecies of *Mytilus* from the pacific coast of Asia *M edulis kussakini* and *M edulis zhirmunskii.*

2.1.1. Species differences defined with Morphology

Investigations on the taxonomy of *Mytilus* have tended to focus on the systematic relationships between *M edulis* and the Mediterranean mussel *M galloprovincialis*. Since the 1860's considerable controversy has surrounded the systematic status of this mussel. While it is regarded by some as a distinct species of *Mytilus*, others consider it merely as a variety of the larger *M edulis* complex (Gosling, 1984). *M galloprovincialis* is believed to have diverged from *M edulis* when Mediterranean Sea was cut off from the Atlantic during a pleistocene age, about 1-2 million years ago (Barsotti and Meluzzi; 1968)

Separation of *M edulis* and *M galloprovincialis* has been based primarily on external shell contours internal features of the shell valves and the colour of the mantle edge. Detailed descriptions of these can be found else where (Verduin, 1979; Gosling, 1984 and references there in, Beaumont *et al.*, 1989)

Using the morphological characters, a number of investigators (Hepper, 1957; Lewis and Seed, 1969; Seed 1972, 1974) have identified the *M galloprovincialis* from on the South-west coasts of England and on the Atlantic coasts of Ireland and France.

Of the six morphological characters used to separate *M edulis* and *M galloprovincialis*, overall shell shape is the least reliable (Seed, 1972, 1974;

Gosling 1984; Beaumont *et al.*,1989)being influenced by environmental factors, the effect of which appear to be the same for both forms of mussel (Seed, 1 978). Of the various internal shell characteristics the anterior adductor scar and hinge plate size have been generally regarded as the two most reliable in separating the two forms of mussel.

Beaumont *et al* (1989) have found mantle edge colour to have good discriminatory power in separating mixed populations of *M edulis* and *M galloprovincialis* in South-west England. However, in other areas eg the Atlantic coasts of France and Ireland and even in the Mediterranean sea, where only *M galloprovincialis* occurs a large percentage (20-60%) of individuals were misidentified using this character alone (Seed, 1972, 1974).

There is no single morphological character that can reliably used to separate mixed or pure populations of the two forms of mussel (Gosling, 1984; Beaumont *et al.*, 1989; Koehn, 1991; Mc Donald *et al.*, 1991).

Ferson *et al* (1985) used an image analysis technique that automatically determines the outlines of shells to discriminate between distinct population of *M edulis* and the recently rediscovered *M trossulus* Gould 1850 (Mc Donald and Koehn, 1988) in New found land , Canada.

Mc Donald *et al* (1991) have used canonical variate analysis of shell traits to discriminate between three different taxa of *Mytilus* collected from sites in the northern and southern hemisphere, prior electrophoretic analysis had indicated that

only one taxon was present at each of the northern hemisphere sites. Eighteen morphometric characters were employed, virtually all of which have been used by previous authors (Seed, 1972; Verduin, 1979; Beaumont *et al* ., 1989) The analysis revealed three clusters in the northern hemisphere, corresponding to *M edulis*, *M galloprovincialis* and *M trossulus*, with the best discrimination between *M edulis* and *M galloprovincialis*. In the southern hemisphere where to date *M trossulus* has not been identified, *M edulis* samples were morphologically intermediate between northern *M edulis* and *M trossulus*. In contrast, both southern and northern *M galloprovincialis* were morphologically similar. For the field taxonomist there is no single morphological character which can be used to discriminate between allopatric populations of *M edulis*, *M galloprovincialis* and *M trossulus* (Gosling, 1992a)

Much of the confusion described above stems from the fact that, until relatively recently, *Mytilus* systematics has been based solely on morphological shell characteristics that are influenced by non-genetic factors such as age and density of mussels, tidal level and habitat type (Seed, 1968; Gosling 1992a). Examination of preserved larval and adult specimens of *Perna perna*, *P viridis* and *P canaliculus* confirmed Seed's (!968) contention that shell shape and thickness are characters of little taxonomic value in the Mytilidae. Variations in shell coloration and patterns are also considerable in all these materials.

Literature dealing with the taxonomy of the green and brown edible mussels of the Indian coasts shows that these are treated invariably under the genus *Mytilus* (Annandale, 1916; Hornell, 1917, 1921; Gravely, 1941; Paul 1942; Jones; 1951; Satyamurty, 1956, Kundu, 1965; Menon, Sareen and Tandon, 1966). However, recently the green and brown mussels hitherto described from the coasts of India as *Mytilus* have been brought under the genus *Perna*. The green mussel recorded and described as *M viridis* by earlier workers has been renamed as *P viridis* while the brown mussel exhibiting distinct morphological traits hence has been assigned the status of a new species *P indica* (Kuriakose & Nair, 1976).

Clearly, systematic information that is relatively free of environmentally induced changes is highly desirable (Seed, 1968). Over the past 20 years, the application of the techniques of protein electrophoresis have enabled in quantifying genetic differences between species (Ferguson, 1980). Electrophoretic technique is an effecient method that separates protein molecules according to their net charge and size. Since each gene controls each protein, analysis of proteins and their variant form can greatly help in detecting genetic variations between and within the species. Thus the total genetic profile of the species can be measured by obtaining the electrophoretic patterns of the general tissue proteins. Thus species identity of even the fish fillets (Shaklee & Keenan 1986), of larvae (Smith & Crossland,1977) and identity of hybrids (Shearer & Mulley 1978, Pullan & Smith 1987) were revealed by gel electrophoresis of tissues proteins / enzymes.

2.12. Species differences defined with Electrophoretic patterns of proteins

Biochemical and molecular systematics have dramatically altered the understanding of the ecology, evolution and biogeography of bay or blue mussels of the genus *Mytilus* (Mc Donald and Koehn, 1988; Koehn, 1991; Seed; 1992; Rawson and Hilbish, 1995).

Protein electrophoresis, together with techniques such as DNA-DNA hybridization mitochondrial DNA analysis, immunology and amino acid sequencing have proved invaluable in quantifying genetic differences between different animal and plant taxa (Gosling, 1992). For differentiation of closely related species electrophoresis has proved to be a most effective technique (Ferguson 1980; Murphy *et al.*, 1990)

Where populations of two forms, came into geographic contact they may hybridize. The size of hybrid zone varys depending on location eg in North West Europe the width of the hybrid zone between *M edulis* and *M galloprovincialis* is large, while that between North sea *M edulis* and Baltic *M trossulus* is narrow, hybridization occurring over a short distance (Vainola and Hvilsom, 1991).

Results from electrophoretic analysis has indicated that such zones are spatially complex, containing a mixture of pure hybrid and introgressed individuals. The *M edulis /galloprovincialis* hybrid zone in north-west Europe has been well

studied (Skibinski and Beardmore, 1979). Over 170 taxa belonging to different animal or plant families are known to be hybrid zone (Barton & Hewitt, 1989). The term hybrid zone is used in a narrower sense when the clines are maintained in a small region by balance between dispersion and selection against hybrids (Barton and Hewitt, 1985)

The genetic structure of populations of the *M edulis* (L) - *M galloprovincialis* (Lmk) complex has been characterised by studying polymorphism by means of isozyme electrophoresis (Viard *et al.*, 1994). In areas of contact, hybridization occurs to such an extent as to contravene the present working definition of species-the biological species concept of Mayr (1970). This defines species as groups of actually or potentially interbreeding individuals, reproductively isolated from other such group and therefore, places heavy reliance on the presence of genetically based barriers to gene exchange between species pairs. Other workers hold a different view point (Mc Donald & Koehn, 1988; Koehn, 1991; Mc Donald *et al.*, 1991) these have tended to concentrate an widely separated allopatric populations of the three forms of mussel, and are therefore stock by the amount of morphological and genetic differences between the different taxa differences which are maintained despite hybridization and the massive potential for larval dispersal. These workers feel that "this genetic distinctness warrants taxonomic recognition at the species level (Mc Donald *et al.*, 1991)

M coruscus and *M californianus* are readily distinguished from other taxa by the presence of radiating ribs on the shell. However, a report by Vermeij (1989) suggests that *M californianus* and *M coruscus* may in fact to be a single species. Mc Donald *et al* (1991) suggest that the South American mussels *M chilensis* and *M platensis* and also *M desolationis* should tentatively be included in *M edulis* and that the Newzealand mussel *M aoteanus* and *M planulatus* from Australia should be in *M galloprovincialis*.

Mc Donald *et al* (1991) have suggested that the two subspecies *M edulis zhurmunski* and *M edulis kussakini;* on the basis of their described geographic distribution (Scarlato and Starobogatov, 1979) should be considered as *M galloprovincialis* and *M trossulus* respectively.

Before the use of electrophoresis, eight *Mytilus* taxa were commonly recognised : *M edulis M galloprovincialis, M planulatus, M platensis M chilensis, M desolationsis, M coruscus* and *M californianus*. The last species is found on the pacific coast of North America, is easily distinguished by the radiating ribs on the shell (Soot-Reyn, 1955). *M coruscus* has been reported from the pacific coast of Asia (Scarlatto, 1981) but since no one has collected allozyme data from this species, its taxonomic status remains obscure. The remaining taxa have been considered races, varieties or sub species of *M edulis* by some authors, while others have considered them full species (Koehn, 1991). The biochemical genetic study carried out by Mc Donald *et al* (1988) concluded that the mussels *M*

galloprovincialis (Lmk) and *M trossulus* are genetically two distinct species. The mediterranean mussel *M galloprovincialis* (Lmk) has been identified on the west coast of southern Africa using morphological and biochemical genetic comparisons with the samples of pure *M edulis* from, Denmark and *M galloprovincialis* from the Mediterranean coast of Spain (Grant *et al.*, 1985) *Mytilus sps* from Sanriku bay Japan were examined using morphological characters and electrophoretically detectable enzyme polymorphisms (Wilkins *et al.*, 1983) and the mussels were identified as *M galloprovincialis*. Skibinski *et al* (1983) used starch gel electrophoresis to study variation at loci in mussels sampled from British coastal silo and two types of mussels such as *M edulis* and *M galloprovincialis* were identified. Microgeographic allozyme differentiation in the hybrid zone of *M galloprovincialis* and *M edulis* on the continental European west coast has been reported by Comesana *et al* (1997). Morphological and genetic differences between Japanese and Chinese red ark shell were examined (Yokogawa, 1997).

2.2. GENETIC VARIATION AT THE LEVEL OF THE INDIVIDUALS

Protein polymorphism have been used to investigate the genetic structure of natural populations of a diverse marine invertebrates (Burton, 1983). Substantial differentiation has been observed in several species that appear to have high dispersal capabilities while some of this differentiation may be the result of natural selection. Other cases (where populations are found to have unique allele in high

frequency) seem to reflect restricted dispersal and gene flow among conspecific population (Burton, 1983)

Electrophoretic studies have been made a total of 8 populations and 400 individuals to determine the amount of genetic diversity within and between populations and taxa (Hedgecock *et al.*, 1984) Electrophoretic analysis of loci controlling a variety of enzymes has been applied to samples of padstow mussel and typical *M edulis* in order to resolve the disputed status of the "pad stow mussel" (Ahmad *et al.*, 1976). Genetic variation exhibited by electrophoretic analysis of tissue proteins and enzymes of the pacific oyster (*C gigas*) was analysed (Buroker *et al.*, 1975). Buroker (1983) studied 19 different geographic population of the American oyster (*C virginica)* by protein electrophoresis. Gartner *et al* (1980) reported the genetic difference among *M edulis* population from four localities in Atlantic Canada using electrophoretic technique. Genetic differentiation were analysed among 18 populations of zebra mussels (*Dreissena polymorpha)* from the Great lakes and seven populations from Europe using starch gel electrophoresis (Marsden *et al.*, 1995). Van-der-Bank, (1995) reported a study on, the allozyme variation in a fresh water mussel population from Southern Africa. Geographic variation in the allozyme frequencies of the brown mussel *(Perna perna)* has been studied by Grant *et al* (1992) in Southern Africa. Starch gel electrophoresis was carried out to survey genetic variation in 25 species of marine mollusc (Fujio *et al.*, 1983) The genetic cohesiveness among populations of the marine gastropod *Concholepas concholepas* from four regions

in Southern Chile were examined (Gallardo *et al.*, 1996). Varvio *et al* (1988) studied the genetic relationships among *Mytilus* populations throughout the North Atlantic region. Eight populations of *M galloprovincialis* from different coasts of the Northern and central Aegean sea have been investigated at the morphological and allozymic level (Karakousis *et al.*, 1993). Pompa *et al* (1990) conducted a study on the genetic variation in four populations of the mussel *Perna perna* from the shores of north east Venezuela. Frank *et al* (1990) reported the enzyme variation between littoral and sub littoral populations of the green-lipped mussel *Perna canaliculus*. Gardner *et al* (1996) reported the biochemical genetic variation among populations of the greenshell mussel *Perna canaliculus* from Newzealand.. Genetic variability has been surveyed in allopatric populations of *M edulis* from over a hundred locations in the northern and southern hemispheres (Levinton and Koehn, 1976; Ahmad *et al.*, 1977; Gratner-kepkay *et al.*, 1980, 1983; Gosling and Wilkins, 1981; Skibinski *et al.*, 1983; Koehn *et al* 1984; Bulnheim and Gosling, 1988; Varvio *et al.*, 1988;. Johannesson *et al.*, 1990; Mc Donald *et al.*, 1990, 1991). Geographic or clinal variation in the frequency of allozyme variants have been observed in European populations of *Mytilus* (Theisen, 1978; Skibinski and Beardmore, 1979; Gosling and Wilkins, 1981). In North America (Koehn *et al.*,1976) and in the Baltic (Theisen, 1978) geographic variation has been initially viewed as occuring within a single species, *M edulis* and has been interpreted interms of natural selection acting at individual allozyme loci; temperature and salinity have been identified as possible selective factors (Koehn

et al., 1980a; Hall, 1985) . In areas eg North West Europe and the east coast USA, South of cape cod, where only pure populations of *M edulis* have been analysed, allele frequencies within each region are remarkably homogeneous over large geographic distances (Ahmad *et al.*, 1977; Gosling 1984; Bulnheim and Gosling, 1988; Varvio *et al.*, 1988; Johanneson *et al.*, 1990; Mc Donald *et al.*, 1990; Vainola and Hvilson, 1991).

Pure populations of *M galloprovincialis* have been analysed electrophoretically. Allele frequencies are fairly homogeneous over large geographic distances (Skibinski *et al.*, 1980, 1983, Wilkins *et al.*, 1983; Grant and Cherry, 1985; Varvio *et al.*, 1988; Beaumont *et al.*, 1989 a; Mc Donald *et al.*, 1990, 1991, Sanjuan *et al.*, 1990) Allele frequencies of *M trossulus*, within single geographic areas tend to be homogeneous (Bulnheim and Gosling, 1988; Mc Donald and Kochn, 1988; Mc Donald *et al.*, 1990, 1991). Gardner *et al* (1996) reported the extent of genetic variation between wild and cultured green mussel *Perna canaliculus*. A similar study was also conducted on abalone, *Haliotis tuberculata* (Mgaya *et al.*, 1995).

Moraga *et al* (1994) reported the genetic differentiation across the western pacific populations of the hydrothermal Vent bivalve *Bathymodiolus spp* and the Eastern pacific population of *Bathymodiothlus thermophilus*. Galleguillos *et al* (1990) reported the protein variation in the scallop *Argopecten purpuratus* and in the mussel *Choromytilus chorus*.

2.3. GENETIC VARIATION BETWEEN THE SPECIES

Buroker *et al* (1979) reported the level of genetic variation for 6 *Crassostrea* and 3 *scaccostrea* species. Skibinski *et al* (1980) studied genetic variation at 13-16 loci in *Modiolus modiolus, M edulis* and *M galloprovincialis*. Gosling (1984) reported that the two forms of mussels *M edulis* and *M galloprovincialis* are closely related, genetic identity and genetic distance value were similar to those observed between sub species of other invertebrates. Bulnheim *et al* (1988) examined the population genetic structure of mussels from various regions of Baltic Sea with reference to *M edulis* and *M galloprovincialis*. Coustauc *et al* (1991) carried out a genetic study on the French Atlantic coast from 1989-1990 revealed interdigitation between typically *M edulis* and typically *M galloprovincialis* populations and intermediate populations. Mc Donald *et al* (1991) reported that the northern and Ssouthern hemisphere samples consists of three electrophoretically distinguishable species such as *M edulis, M galloprovincialis* and *M trossulus*.

The electrophoretic profiles of proteins / enzymes were also used in diffenentiating the Indian species of prawns (Thomas, 1981., Philip Samuel, 1981) crabs (Kannupandi & Paulpandian, 1975) fishes (Manohar & Velankar, 1973., Menezes, 1979., Mahobia,1987.,Chakraborty 1990). However, application of electrophoretic techniques in differentiating the Indian Bivalves like mussels was not reported earlier.

The essence of the above review is that all tissue proteins/ enzymes are potential genetic markers and any one or more markers may be selected and analysed by electrophoretic method to study the genetic variability between the species, within the species, among the populations and also to verify whether the suspected individual is a hybrid or a new species. The present investigation on the electrophoretic profiles of the general proteins and morphometrics in the Indian mussels was aimed at detecting the genetic differences between the green mussel *P.viridis* and the brown mussel *P.indica*. The results and the conclusions of the investigation are presented in this dissertation.

3. Materials and Methods

3.1. MATERIALS

3.1.2. COLLECTION

Wild specimens of the green mussels (plate 1) were collected from Calicut (11°15'N 76° 45'E), Cochin (9^0 58'N 76^0 16'E), Quilon ($9^0$57'N 76^0 33'E), Vizhinjam (8° 22'M, 76° 56E) and Madras (13^0 06'N 80^0 18'E), (fig-2) wild specimens of brown mussels (plate 2) were collected from Cochin Vizhinjam and Mandapam (9^0 16'N 79^0 12'E) fig (2). The samples collected from Mandapam were the stock from Vizhinjam. Two types of suspected hybrids were collected from the Vizhinjam (plate 3) and Cochin (plate 4). The suspected hybrids of Vizhinjam were present along with the green and brown mussel population where as the suspected hybrids of Cochin were collected from the onshore areas of the Narakal without the interference of brown and green mussel population. The green mussels were collected from the Fort Kochi and brown mussels were collected from the Chellanam area of the Cochin. A total of 10 green, 10 brown and 10 suspected 'green type' hybrids were collected from Cochin. A total of 21 green mussels, 20 brown mussels and 8 number of suspected 'brown type' hybrid mussels were collected from Vizhinjam. Likewise, 20 green mussels were also collected from the back water areas of Quilon and 20 number of green mussels were collected from the Calicut. 21 number of green mussels and 10 number of brown mussels were also collected from Madras and Mandapam respectively.

Thus a total of 150 specimens were examined from the five regions.

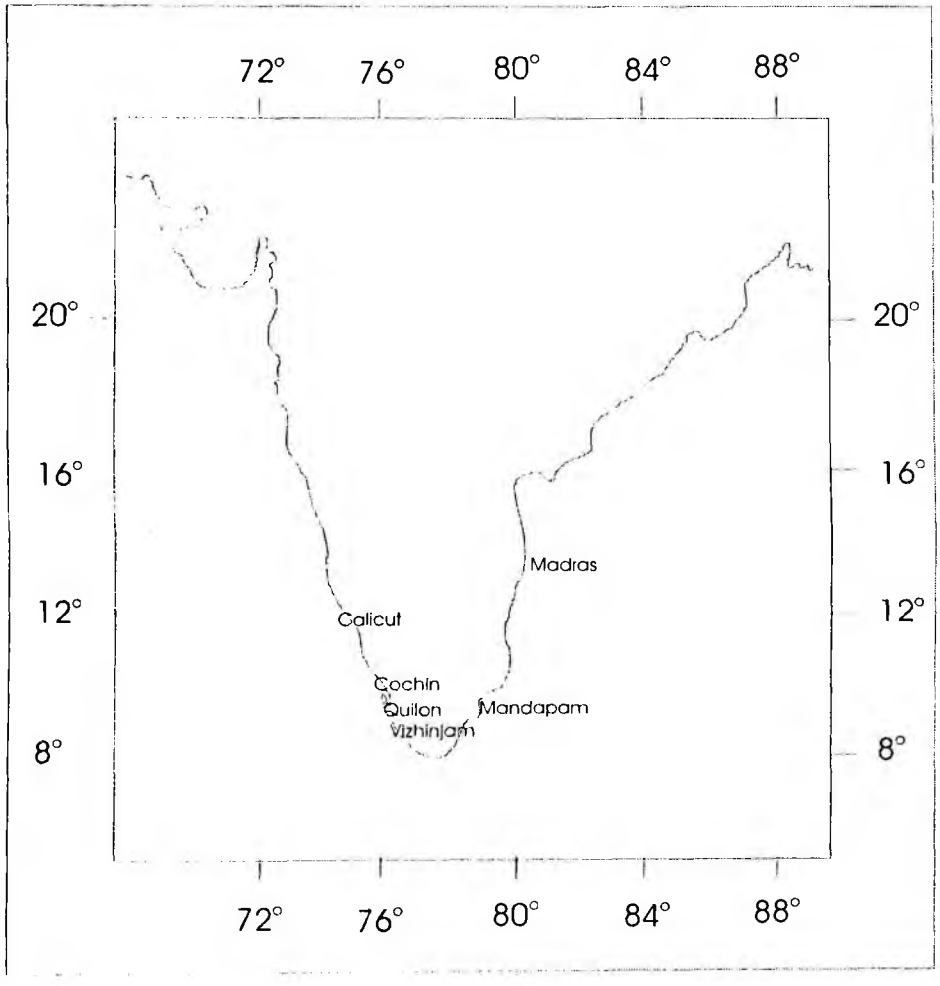


Fig.2. Collection sites of *P viridis* and *P indica*

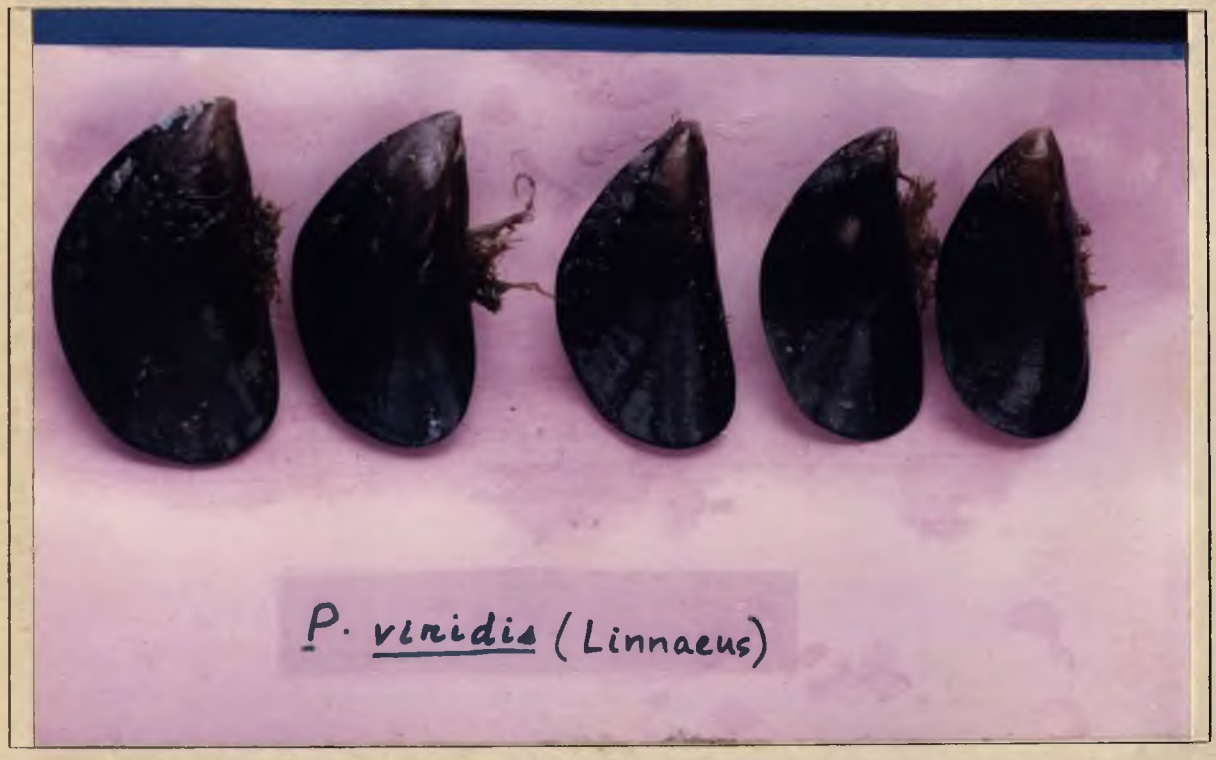


Plate 1. Green Mussel. (*Perna viridis*)

Plate 2. Brown mussel (*Perna indica*)

Plate 3. Suspected 'brown type' hybrid mussel

Plate 4. Suspected 'green type' hybrid mussel

3.1.3. TRANSPORTATION :

The specimens from the different collection centres were transported in live condition to the lab. Within 7 hours specimens were brought to the lab except from Madras and Mandapam. All the samples were transported live in moist condition. From Madras the samples were brought in moist condition to the Calicut Research centre of CMFRI and there the animals were transferred to seawater and aeration was provided. On the same day, after 3-4 hours mussels were again kept in moist condition and transported to Cochin. In the lab, animals were properly cleaned in seawater and were immediately stored at —20°C, till analysis.

Similarly, the samples (brown mussels) were collected from Mandapam and brought to lab via Vizhinjam Research centre of CMFRI.

3.2. METHODS

3.2.1. SPECIES IDENTIFICATION BASED ON MORPHOLOGICAL CHARACTERS

Both green (*P viridis*) and brown (*P indica*) mussels were differentiated based on their morphological characters (Kuriakose *et al.*, 1976). The diagnostic characters separating the species *P viridis* and *P indica* are given in Table 1. The morphological characters of the suspected intermediate individuals collected from Vizhinjam and Cochin were examined with respect to the characters of green and brown mussel.

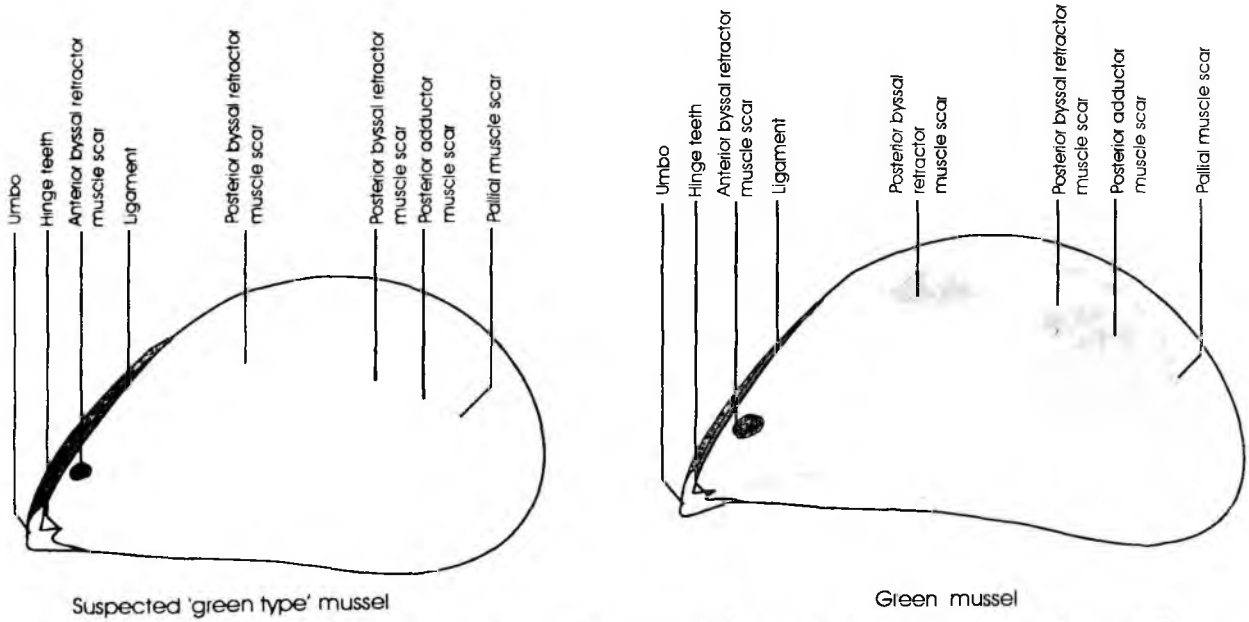


Fig.4. Internal view of right valve of mussels showing muscle impressions, ligament and hinge teeth.

Suspected 'brown type' mussel

Brown mussel

3.2.2. MORPHOMETRICS :

Dorso Ventral length (DVL), shell width, shell thickness (Fig 3) of each individual were measured (Table 4) and the average value of each parameter was calculated. Morphometric comparison between the green mussels from different regions, between green and brown mussels, between green, brown and suspected green/brown type mussels was done.

3.2.2. a. Discriminent analysis for grouping of suspected mussels.

Discriminent Analysis was done according to the method described by Rao (1965) for grouping of the suspected individuals collected from Cochin and Vizhinjam using SPSS software.

Morphometric meassurements of a total of 93 mussels (63 green and 30 brwon) (Table 6) were taken and the discriminent function was calculated. Using the fitted discriminent function for discriminating green and brown mussels, the discriminent scores of each suspected individuals was calculated. Based on these scores a rule for classification to one of the two groups were formulated based on posterior probability as follwed.

(i) Get an estimate of prior probability that an animal belong to group 'i' as P (Gi) from their availability in the population.

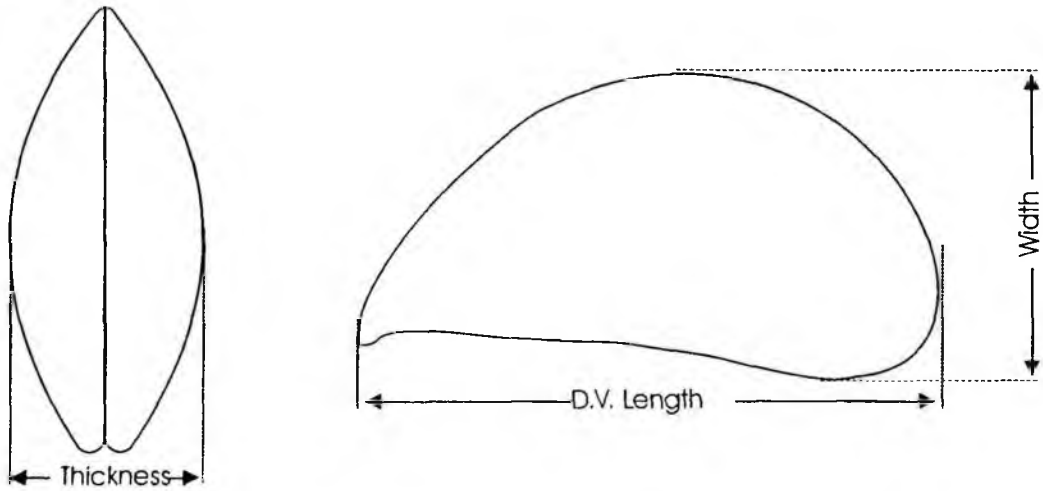


Fig.3. Various morphometric measurements taken for mussels

(ii) Using the means and standard deviations of scores of the groups assuming normality,. compute the conditional probability P (D/Gi)

(iii) Using Baye's formula workout the posterior probabilities as

$$P(Gi/D) = \frac{P(D/Gi)\ P(Gi)}{\sum_{i=1}^{2} P(D/Gi)\ P(Gi)}$$

(iv) Group the animal with discriminant score D to that group for which P (Gi/D) is the largest.

The percentage of green mussel in Vizhinjam = 5 %

The percentage of brown mussel in Vizhinjam = 95%

Hence $P(G_1) = 0.05$

$P(G_2) = 0.95$

The percentage of green mussel in Cochin = 80%

The percentage of brown mussel in Cochin = 20%

Hence $P(G_1) = 0.80$

$P(G_2) = 0.20$

3.2.2. b. Principal component analysis

To see whether there is any geographical difference in green mussel populations, principal component analysis was carried using the morphometric

measurement. The Ist two PC's explained percentage of variation. Morphometric measurements were first transformed by natural logarthims and the transformed value were used for PC analysis on the sum of squares and sum of products matrix.

One way analysis of variation was done to see significant difference between centres.

3.2.3. BIOCHEMICAL GENETICS

3.2.3.1. ELECTROPHORETIC ANALYSIS

3.2.3.1.1. STANDARDISATION OF THE METHODOLOGY

3.2.3.1.2. a Sample Preparation.

Tissue samples from adductor muscle, mantle, foot, gill and digestive diverticula were taken from the thawed mussels in cold conditions. Each of these was weighed and packed in aluminium foil, labelled properly and was immediately kept below 0°C until use. This procedure was repeated initially for five individuals at a stretch. Later, each of the tissue was minced under cold conditions and then separately homogenised in selected media at selected tissue medium ratios. The homogenising media tried were double distilled water (DDW) and 0.05M Tris/HCl, (pH 7) buffer. The ratios at which the media were utilised for homogenising the three selected tissues were 1:1 (w/v), 1:2 (w/v) and 2:1 (w/v). Mechanical homogeniser (Remi) and a manual glass homogeniser were employed. Homogenisation was invariably conducted under cold conditions. Homogenates

were taken in Eppendorf tubes. Centrifugation was done at speeds ranging from 5,000 to 10,000 rpm for periods ranging from 5 minutes to 30 minutes at 4.°C. (These conditions were selected based on the experience of previous workers on other bivalves). Supernatant obtained was drawn and transferred to another set of labelled Eppendorf tubes and stored at —20°C till they were analysed

3.2.3.1.1.b. Reagents for stock solutions:

1. Gel buffer (Tris -HCl)

   a. Separating gel buffer (Tris - HCl) 1.8M pH 8.9

      Tris -10.9 gram for 50ml.

   b. Stacking gel buffer (Tris - HCl) 0.5M pH - 6.8

      Tris - 3.02 gram for 50 ml

2. Tank buffer (Tris Glycine) 0.2M pH 8.3

   Tris Glycine-36.03 grm for 2.4 litre.

All the buffer reagents were dissolved separately in double distilled water and made upto the required volume. The pH was checked with digital pH meter. The pH of gel buffer was adjusted adding 8M HCl as required. The pH of tank buffer was adjusted by adding 2M Tris (Stock).

3. 30%. Acrylamide Solution:

   30 grams of acrylamide was dissolved in 100ml of DDW (w/v) and filtered through whatman filter paper. The solution was kept at 4°C in an amber coloured bottle.

4. 2.5% Bisacrylamide solution:

2.5 grams of bisacrylamide was dissolved in 100 ml DDW (w/v) and filtered through whatman filter paper. The solution was stored at 4°C in an amber coloured bottle.

5. Loading buffer :

Loading buffer consisted of 1ml of 0.5 % Bromophenol Blue, 2ml of Glycerol and 7ml DDW. At first the required quantity of glycerol and buffer were mixed and then dye was added to it.

6. APS : 5% APS solution prepared on the same day of electrophoretic run.

0.05 gm of APS was dissolved in cold DDW and stored at 4°C until use.

3.2.3.1.2.c. Electrophoresis:

Electrophoresis was done in horizontal electrophoretic unit. Polyacrylamide gel medium was used. Various proportions of acrylamide (30% stock) and Bisacrylamide (2.5% stock) solutions were tried to maximise the resolution and separation of bands. However, for any gel percentage the proportion of Bisacrylamide was 5% of the total acrylamide concentration (Gordon, 1980) For one gel, 50 ml of separating gel and 25ml of stacking gel was prepared. The amount of buffer, APS, TEMED in any percentage of gel was kept constant. 350

µl APS and 50µl TEMED were added for separating gel where as half of each respective quantity was used for stacking gel preparation.

The proportion tried for sample and loading buffer were 1:1 (v/v), 2:1 (v/v) and 1:2 (v/v). A total volume (sample + dye) of 60 µl were loaded in each well.

Electrophoresis was stopped when the marker dye reached the anodal end, which usually occured within 3 hours.When electrophoretic run was over, the gel was taken from the cassette and stained for general proteins.

The best tissue giving maximum activity, number of bands and showing band variation for a given set of electrophoretic conditions, producing the best separation and resolution of bands was selected for protein analysis. The composition of the gels used are given in Table 2.

The electrophoretic profile of two enzymes such as Esterase (EST) and Malate dehydrogenase (MDH) were also studied. The method standardised for protein separation was utilised for the enzyme systems. The staining method of Shaw and Prasad (1970) was adopted for detection of enzymes. The electrophoretic banding patterns obtained after the staining were recorded as well as photographed for further reference and analysis.

3.2.3.2. General protein and Enzyme staining Recipes:

1. General protein :

Monomer

Coommasie Blue (SRL) 1.25g

Methanol	230ml
DDW	230ml
Glacial Acetic acid	40ml

Filter the solution. Stain the gel in dark for 90 minutes and coasts. Transfer to destaining solution containing 150 ml of methanol, 70 ml of Acetic acid and 780 ml of water.

2. Esterase

Substrate

1% α, β-naphthyl acetate

2-Naphthyl acetate	1g
β-Naphthyl acetate	1g
Acetone	50ml
DDW	50ml

Stain

Fast blue RR	100 mg
0.5 M tris - Hcl, pH 7.1	100ml
1% α, β-naphthyl acetate	3 ml
DDW	87 ml

Incubate at room temperature until blue bands appear wash and fix.

3. MDH. For 100 ml

2M Tris HCl (pH 8.0)	80 ml
2M D-L Malic acid (pH 7.0)	12 ml
Nicotinamide Adenine Dinucleotide (NAD)	4ml
Nitro- blue Tetra solium (NBT)	3ml
When ready to stain add :	
Phenazonium metho sulfate (PMS)	1.2 ml

Incubate at 37 ° C in dark until sufficient activity is present.

3.2.3.3. PROCEDURE FOR ANALYSIS OF DATA:

3.2.3.3.a. Interpetation of Zymogram patterns :

The electrophoretic position of the major and minor protein/enzyme fractions obtained in the green and brown mussels were closely examined to detect regions that differentiated the two species. The same method was adopted to examine the suspected hybrids. To study the genetic variations within the green mussel,protein profiles between the green mussels were examined to detect polymorphic phenotypes at assumed loci. The phenotype variants at an assumed locus were designed as slow moving S band (slow homozygote), fast moving F band (fast

homozygote)and their combination as SF band (hetrozygote), depending on the distance migrated by the particular band in that particular gel area. As a standard practice, the observed protein phenotypes are presumed as genotypes produced by co-dominant alleles at a particular genetic locus.

The number of gene loci controlling the observed phenotypes in the two species were also estimated on the basis of gene-protein relationship. Thus it was pressumed that each protein fraction in a product of a particular gene in each species. The fastest migrated band was designated as band number one and gene locus one. The subsequent bands loci down towards the point of application of protein extract were given higher serial numbers. Differences in the migration of the bands were the basis of counting the number of loci (Fig. 6).

Table 1. Diagnostic characters separating the species of *Perna.*

Diagnostic Characters	*P viridis*	*P indica*
Shape of anterior end	Pointed, beak - like, downturned	Pointed and Straight
Size of hinge plate	Thick, broad, extends slightly to the ventral border	Thick, narrow, terminal
Number and Size of hinge teeth	Two small on the left valve and one on the right valve	One large on the left valve and a corresponding depression on the right valve
Dorsal ligamental margin	Curved	Straight
Mid-dorsal shell	Arcuate	A distinct dorsal angle or hump present
Ventral shell margin	Highly concave	Almost straight
Mantle margin colour	Yellowish green	Brown
Excurrent aperture opening	Mouth oval and wide; passage into the mantle cavity small; restricted by rectum and rectum and posterior adductor not visible through the opening	Mouth and passage into the mantle cavity are of same width; rectum and posterior adductor prominently visible through the opening.
Ventral mantle margin	Inner fold of the posterior ventral mantle margin thin, extensible, smooth, tentacles or papillae absent.	Inner fold of the posterior mantle margin very thick not extensible provided with 18-22 thick branching tentacles.
Posterior byssal retractors	Two, short, thick bundles; anterior bundle arises from the posterior and diverges in the form of a 'V'.	Two, short, thick bundles; anterior bundle arises from the posterior and diverges in the form of a 'V'.

Source :CMFRI bulletin 29

Table 2. Compositon of gels used in the electrophoretic analysis in mussels

Gel composites	GEL COMPOSITION							
	7% gel		8% gel		9% gel		10% gel	
	Separating gel (50 ml)	Stacking gel (25 ml)	Separating gel (50ml)	Stacking gel (25 ml)	Separating gel (50ml)	Stacking gel (25 ml)	Separating gel (50ml)	Stacking gel (25ml)
Acrylamide(30%)	11.07	2.75	12.65	3.2	14.1	3.5	15.68	3.9
Bis acrylamide (2.5%) Buffer	7	1.75	8	2	9	2.25	10	2.5
(a) 1.8M Tris pH 8.9	10	-	10	-	10	-	10	-
(b)0.5 M Tris pH 6.8	-	5	-	5	-	5	-	5
TEMED	50*	25*	50*	25*	50*	25*	50*	25*
APS (5%)	0.35	0.175	0.35	0.175	0.35	0.175	0.35	0.175
DDW	21.6	15.32	19	14.6	16.5	14.05	13.92	13.4

* in microlitre

4. Results

4.1. STANDARDISATION OF METHODOLOGY:

To select a suitable homogenising medium, double distilled water, 0.2 M sucrose solution and 0.05M Tris/HCl buffer (pH-7), were tested and the results were compared. TrisHCl, buffer (pH-7) at 2:1(w/v) ratio followed by centrifugation at 10,000 rpm for 15 minutes at 4°C gave satisfactory results.

Out of the different polyacrylamide gel percentage examined, better resolution and clarity were obtained at 9% gel for general proteins of adductor muscle, mantle, foot and gill tissues. However, for enzymes gill and adductor muscle gave better result at 8% gel concentration.

4.2. THE ELECTROPHORETIC PROTEIN PROFILE OF THE GREEN MUSSEL (*P.viridis*)

4.2.1. Protein banding patterns in the tissues.

The general proteins extracted from the tissues, mantle, adductor muscle, foot and gill were separately obtained by gel electrophoresis. Each tissue showed its own specific banding patterns. The comparative gel position of the major and minor bands among the four tissues differed significantly indicating tissue specific nature of the proteins in the species. (Fig .6)

The tissue specific differences were also due to differences in the size of some bands and also their staining intensity. The total number of the bands was not different among the tissues. For example, the total number of bands in the

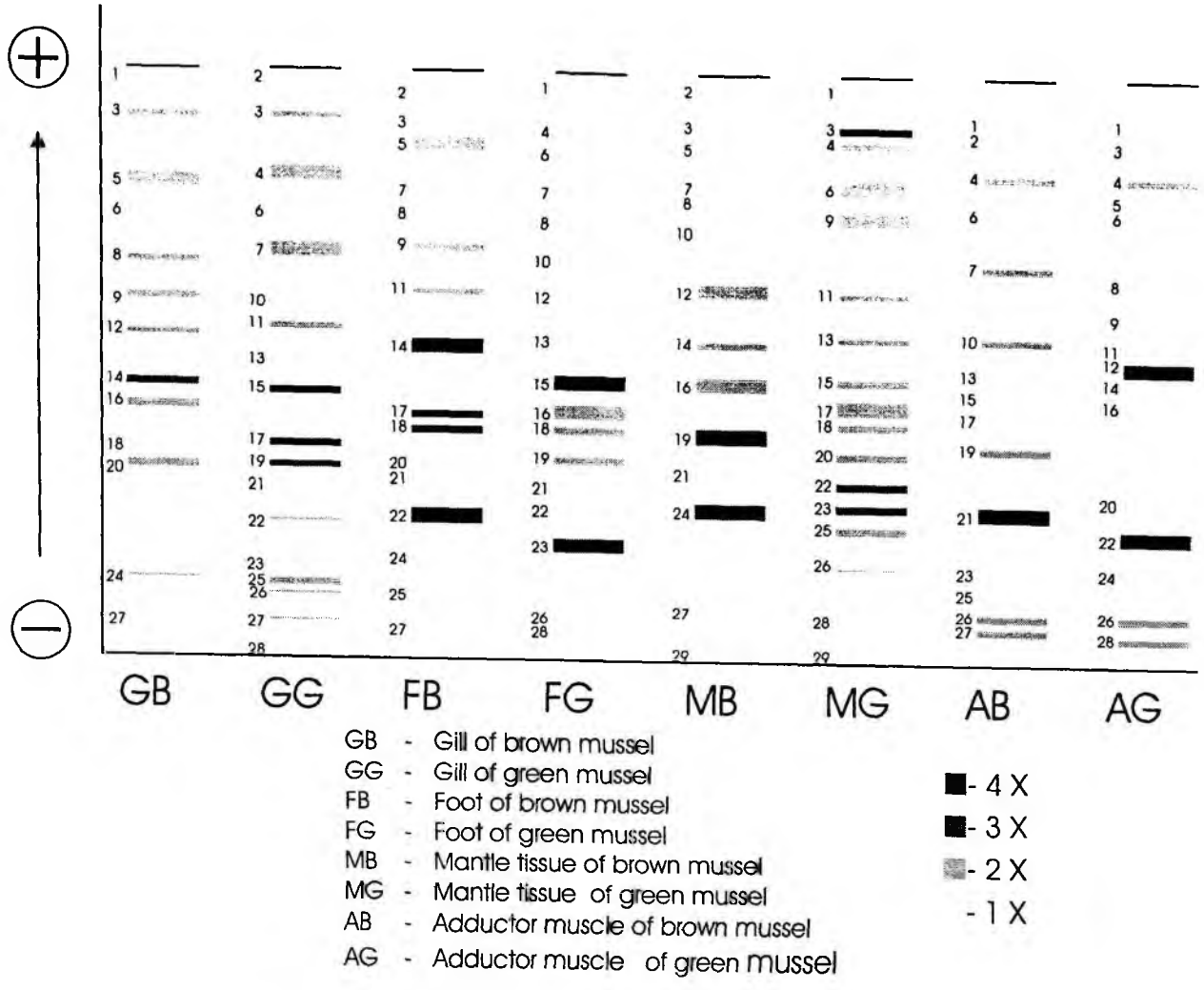


Fig.6. Zymogram of general protein in different tissues of green and brown mussels

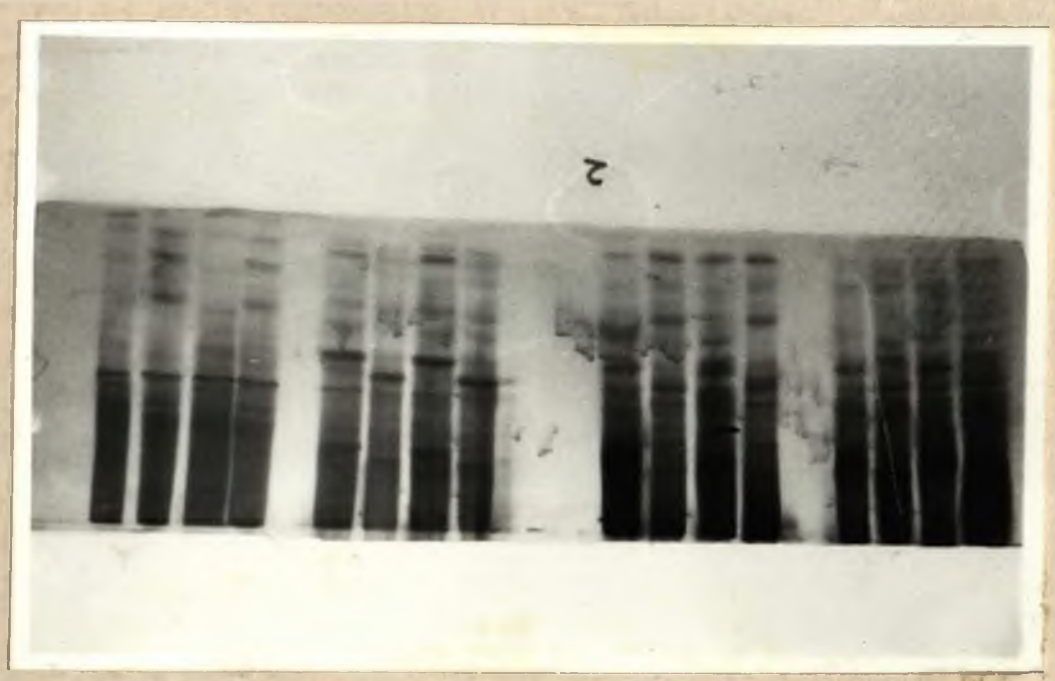


Plate 5. Species specific protein profiles in diffeent tiissues of brown and green mussels.

(L to R : Lanes 1 to 4 : Gill, Lanes 6 to 9 : Foot, Lanes 12 to 15 : Mantle, Lanes 17 to 20 : Adductor muscle)

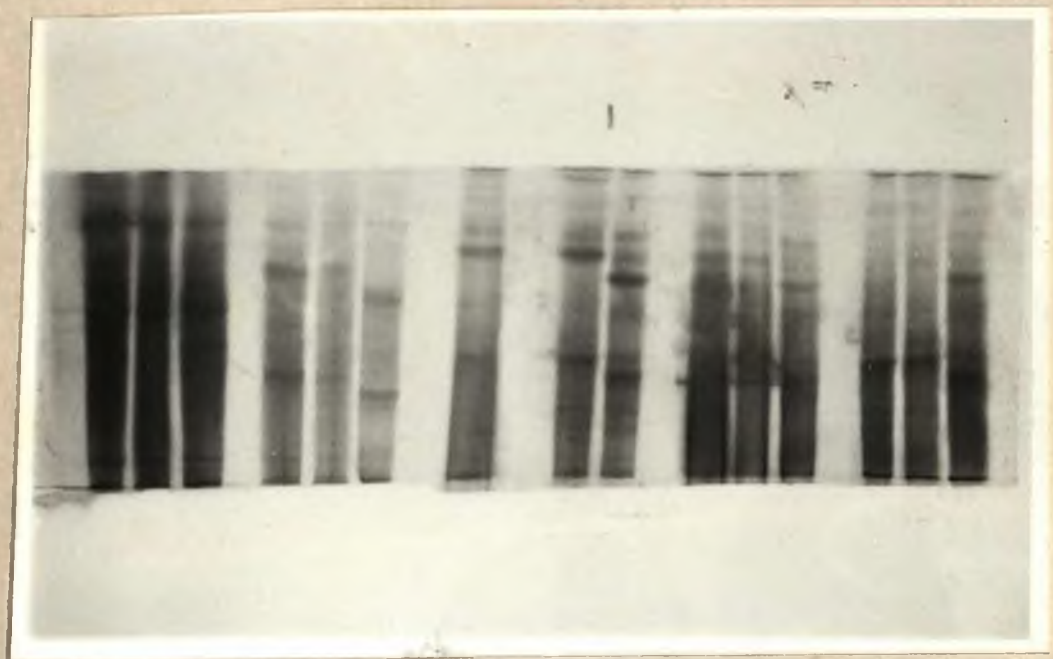


Plate 6. Protein profiiles in different tissues of brown 'brown type' hybrid and green, mussels. The patterns were similar in the brown and 'brown type' hybrid whereas different in the green and brown.

(L to R : Lanes 1 to 3 : Digestive diverticula, Lanes 5 to 7 : Gill, Lanes 9 to 12 : Foot, Lanes 14 to 16 : Mantle, Lanes 18 to 20 : Adductor muscle)

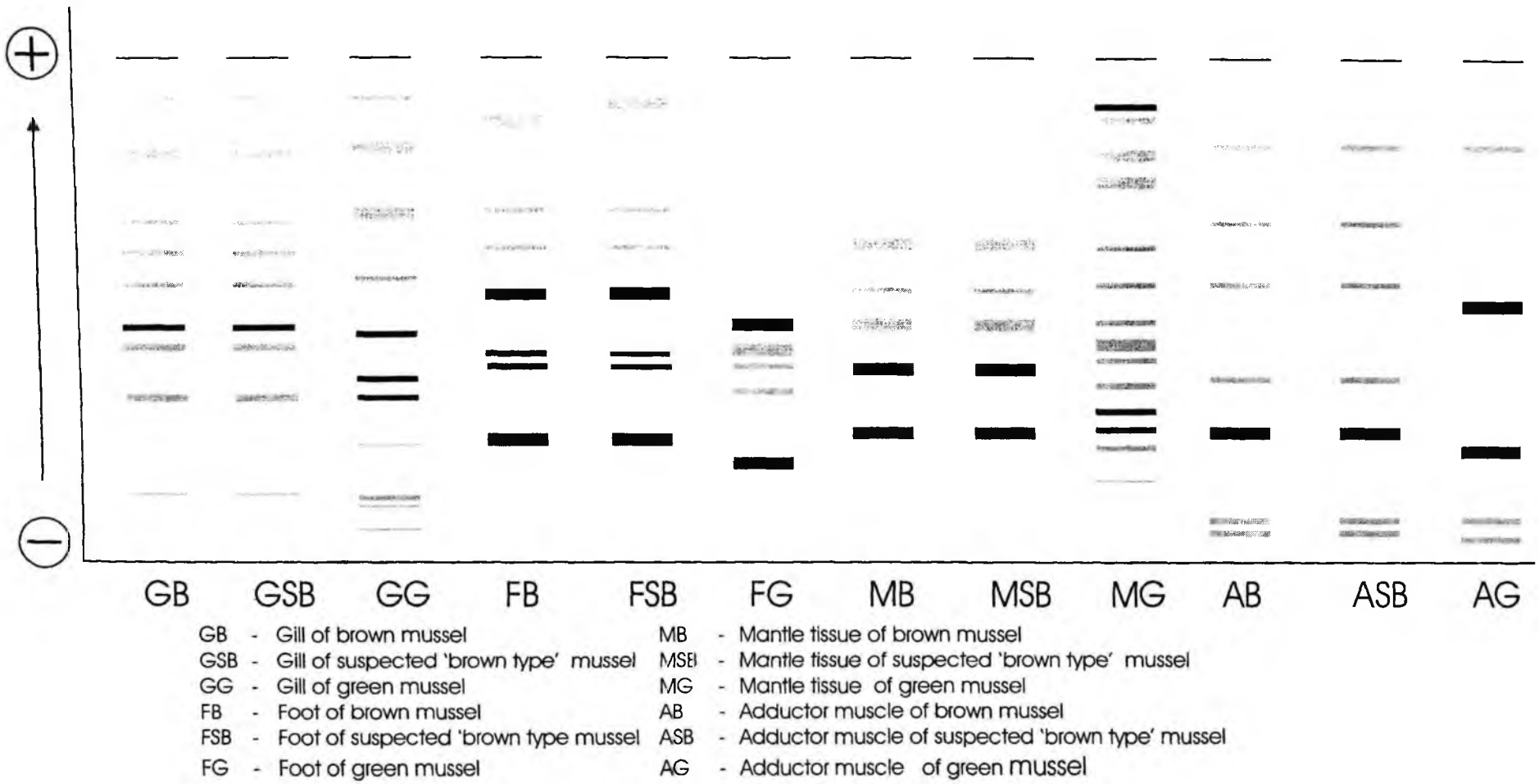


Fig.7. Zymogram of general protein in different tissues of green, brown and suspected 'brown type' hybrid mussels

Plate 7. The protein profiles in the green and 'green type' hybrids are simlar in the mantle tssue.
(L to R : Lanes 1 to 7 & 14 to 20 : Green, Lanes 9 to 12 : Green type.

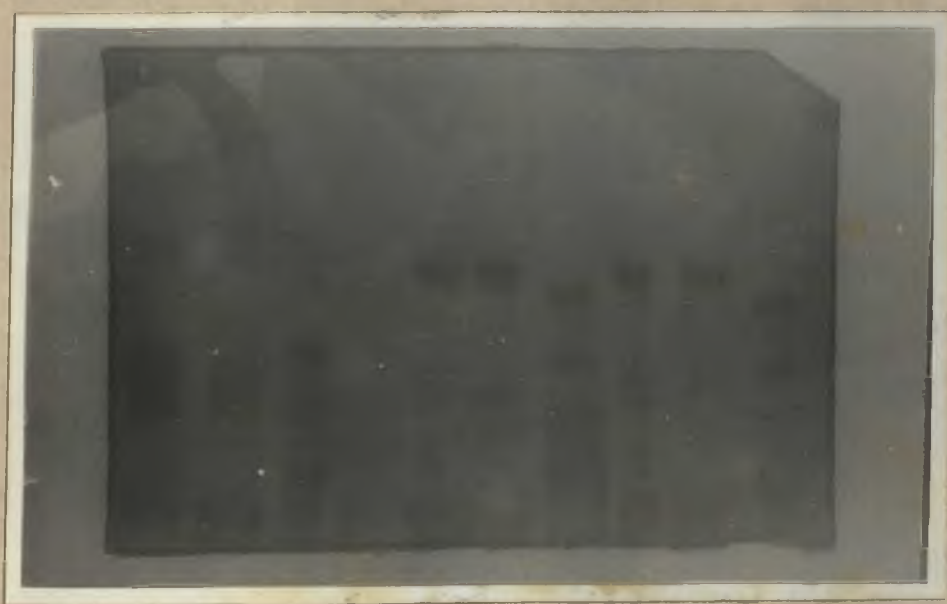


Plate 8. The esterase enzyme profiles in the adductor muscle were similar in the brown and 'brown type' hybrids mussels but different in the green mussel.
(L to R : Lanes 5, 8 : Brown, Lanes 6, 9 : Suspected 'brown type', Lanes 7,10 Green).

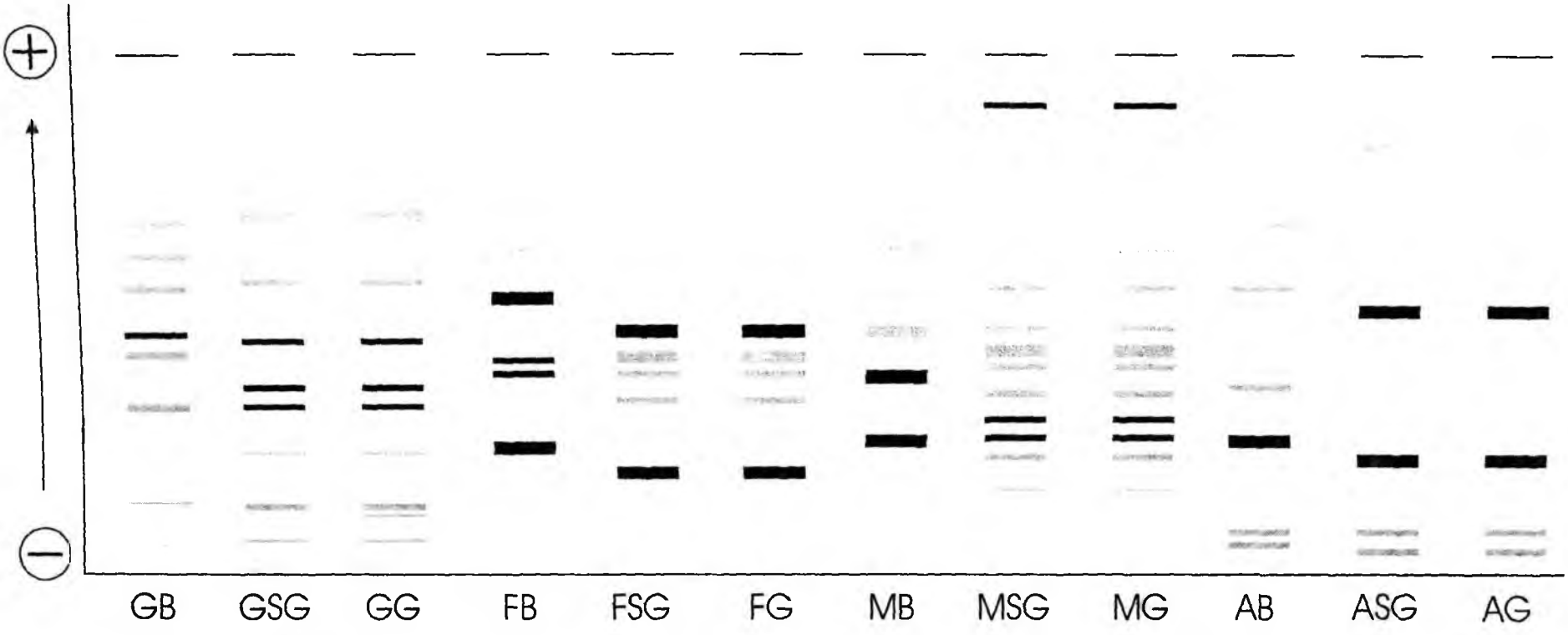
adductor muscle, mantle, foot and the giill was 28,29,28 and 28 respectively. However, the number of tissue bands in different individuals may vary by one or two bands. Though the number of bands in the tissues were almost similar, the differences in the gel position, size and staining intensity of major/minor bands indicated significant tissue specific protein profile in these four tissues. (Fig.6).

4.2.2. Protein profile in the sample populations.

A comparison of protein banding patterns in the green mussel samples collected from Vizhinijam, Quilon, Cochin, Calicut and Madras was made to detect the intraspecies genetic variations,if present, in the species. The banding patterns in some of the ten specimens showed variation at certain protein zones. Most of these variations were due to difference in the number of bands, staining intensities or even the absence of bands. Some of the such variations were also differed between regional samples. The observed individual variations at the particular protein zones appeared to the inconsistent.

4.3. PROTEIN PROFILE IN THE SUSPECTED "GREEN TYPE HYBRID"

The protein profiles of the green mussel *(P.viridis)* and the specimens suspected to be a hybrid of the green and brown mussel were compared. Morphologically, the suspected variety differed from that of the green mussel in having brown coloured shell top with green coloured edges at the posterior end.



GB - Gill of brown mussel
GSG - Gill of suspected 'green type' mussel
GG - Gill of green mussel
FB - Foot of brown mussel
FSG - Foot of suspected 'green type mussel
FG - Foot of green mussel
MB - Mantle tissue of brown mussel
MSG - Mantle tissue of suspected 'green type' mussel
MG - Mantle tissue of green mussel
AB - Adductor muscle of brown mussel
ASG - Adductor muscle of suspected 'green type' mussel
AG - Adductor muscle of green mussel

Fig.8. Zymogram of general proteins in different tissues of green, brown and suspected 'green type' hybrid mussels

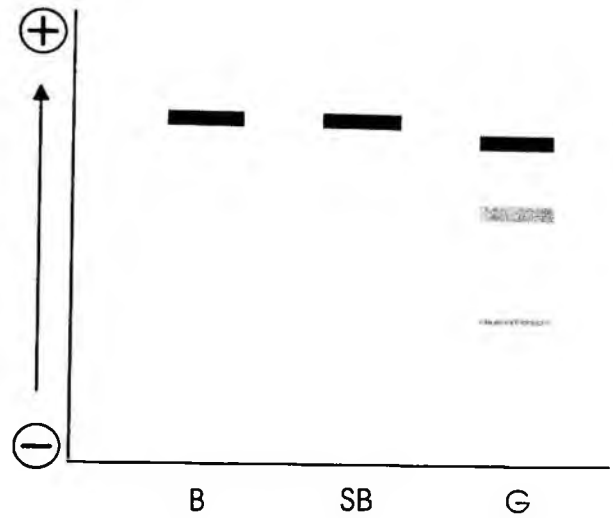


Fig.9. Zymogram of the esterase enzyme profiles in the adductor muscle of the green, brown and suspected 'brown type' hybrid mussels

B - Brown mussel
G - Green mussel
SB - Suspected 'brown type' mussel

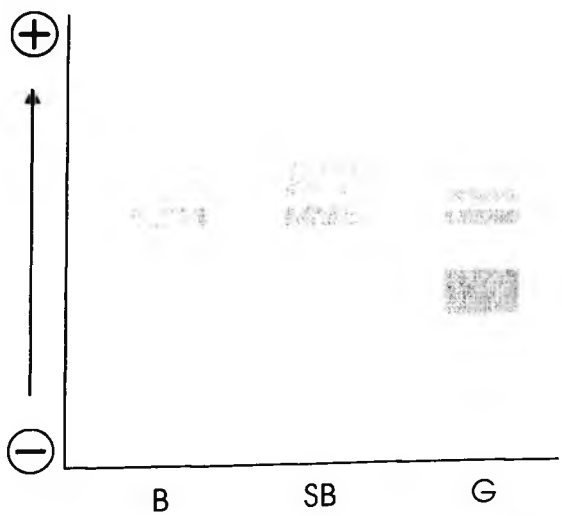


Fig.10. Zymogram of the MDH enzyme profiles in the adductor muscle of the green, brown and suspected 'brown type' hybrid mussels

The protein banding patterns of the adductor, mantle,gill and foot tissues in both the suspected and the wild green were almost similar in spite of the shell colour differences (Fig.8, Table 11).

4.4. THE ELECTROPHORETIC PROTEIN PROFILE OF THE BROWN MUSSEL (*Perna indica*)

4.4.1. Protein banding patterns in the tissues.

The total number of protein fractions in the mantle, foot, adductor and gill tissues was 29, 27, 27and 26 respectively. However, the number of bands present in each tissues was found to vary by one or two in different specimens tested. Irrespective of the number of bands present in different tissues, each tissue had shown its characteristic bands indicating tissue specific protein profile. Tissue specificity of the bands was expressed by having different electrophoretic migration of the concerned bands and thus located in different positions on the gel. (Fig.6, Table 10) Besides, the size and staining intensity of certain bands in each tissue also differed.

4.5. PROTEIN PROFILE IN THE SUSPECTED "BROWN TYPE" HYBRID

An electrophoregram of the general proteins in the adductor muscle, mantle, foot and gill and that of the enzymes, malate dehydrogenase and esterase present in

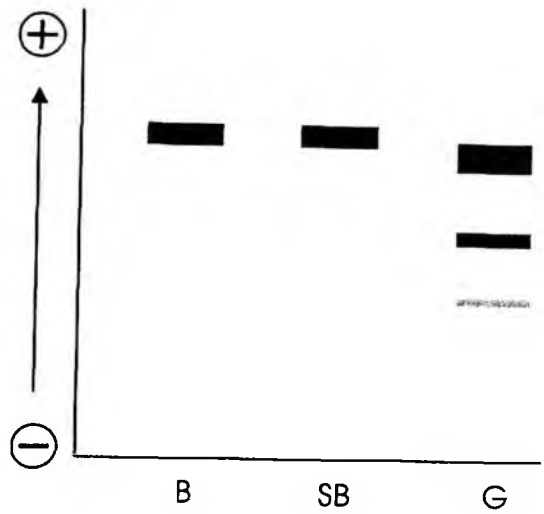


Fig.11. Zymogram of the esterase enzyme profiles in the mantle tissue of the green, brown and suspected 'brown type' hybrid mussels

B - Brown mussel
G - Green mussel
SB - Suspected 'brown type' mussel

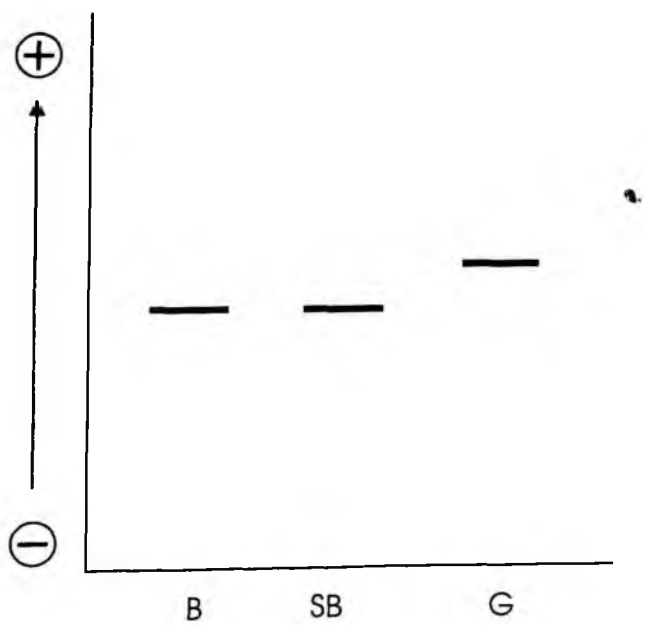


Fig.12. Zymogram of EST enzyme profiles in the gill tissues of green, brown and suspected 'brown type' hybrid mussels

Plate 9. The esterase enzyme profiles in the gill tissue of brown and brown type hybrid are simlar whereas these were different in green and the brown
(L to R : Lanes 1,4,7 : Brown, Lanes 2,5,8 : Suspected 'brown type', 3,6,9 : Green)

Plate 10. The MDH enzyme profiles in the brown and brown type hybrid mussels were similar but it was different in the green mussel.
(L to R : Lanes 1,4,7 : Brown, 2,5,8, Suspected 'brown type' Lanes 3,6,9 : Green).

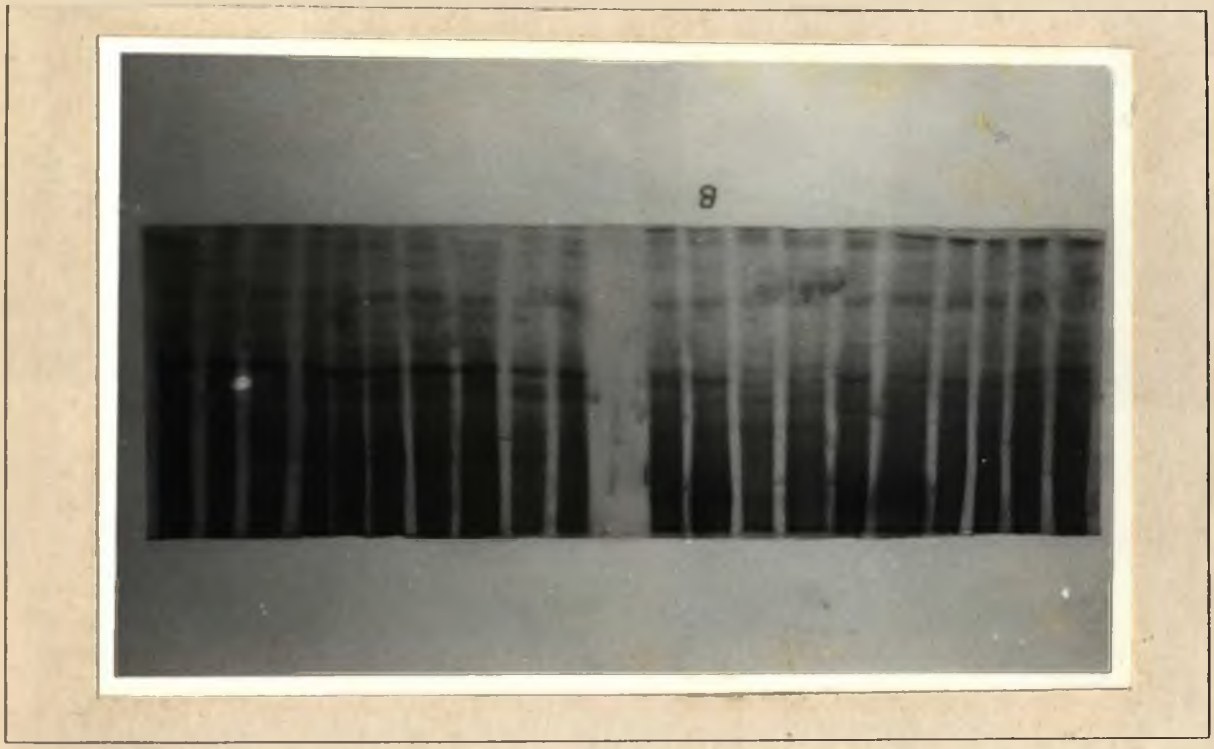


Plate 11. The protein profiles in the green mussel sample with some inconsitent variations in the mantle and foot tissues.
(L to R : Lanes 1 to 10 : Foot, Lanes 12 to 21 : Mantle)

the green , brown and the suspected " brown type" hybrid were obtained (Plate 6, Fig 7, Table 10).

A comparison of the protein and the enzyme profiles of these hybrid types of mussels with that of the brown mussle clearly showed that the protein/enzyme banding patterns in the brown and the suspected hybrid mussels are almost identical whereas the green mussel showed its species specific pattern as described earlier. Though the suspected hybrid has a shell shape of the brown and the green colour of the green mussels, its protein/enzyme banding patterns are that of the brown mussel.

4.6 . THE COMPARATIVE PROTEIN/ENZYME PROFILES IN THE GREEN (*P. VIRIDIS*) AND THE BROWN (*P. INDICA*) MUSSELS.

A comparison of the protein profiles present in the tissues, adductor muscle, mantle, foot and gill of the green and brown mussels showed species specific major and minor proteins fractions. The species specific differences in the protein profile are due to differences in the electrophoretic variations of the major/minor fractions as well as due to differences in the size and staining intensity of certain bands. The species specific difference between the green and brown mussels was most strikingly expressed by a very intensively stained major band present in the foot tissue and located on the gel at 5 to 5.3cm in the green mussel while at 5.7 - 6.0 cms in the brown mussel . The species specific protein fractions are shown as

number 14 in the brown and 15 in the green mussels (Fig .6) . Similarly the banding patterns of the enzymes malate dehydrogenase (MDH) and the esterase (EST) were also distinctly different in both the species (Fig 9, 10, 11, 12, Table 8, 9)

A total of 29 loci in the mantle, 28 loci each in the foot, the adductor muscle and the gill were identified and numbered in the two species. The species specific distinct protein loci in the mantle tissue of the brown mussel were 8, 10, 14, 19, 21, 24 and 27 whereas such unique loci in the green mussel were 3, 4, 9, 13, 17, 18, 20, 22, 25, 26, 28 (Fig 6). In the foot tissue, the species specific loci in the brown mussel were, 1,3,5, where as such specific loci in the green mussel were 2,4,5,9,14,20,22,24,25,27. This species specific loci in the adductor muscle of the brown mussel were 2, 6, 7, 10, 13, 15, 17, 19, 21, 25 and 3, 5, 8, 9, 12, 14, 16, 20, 22, 28 in the green mussel. Of all the loci, the locus in the foot numbered 14 in the brown and 15 in the green mussels have produced intensly stained major bands differentiating the two species easily at one glance. The species specific loci in the gill tissue of the brown mussel were the numbers 1, 5, 8, 9, 12, 14, 16, 18, 24, 26 where as such specific bands in the green mussel were 2, 4, 7, 10, 11, 13, 15, 17, 21, 22, 23, 25, 26, 28. The species specific enzyme loci (MDH) were 2, 4, 5 in the green mussel whereas these were 1,3 in the brown mussel. The specis specifc esterase (EST) enzyme loci in the adductor muscle of the green mussel were 2, 3, 5, 6, 4, 9 whereas these were 1,4,8,10 in the brown mussel.

4.7. MORPHOMETRICS

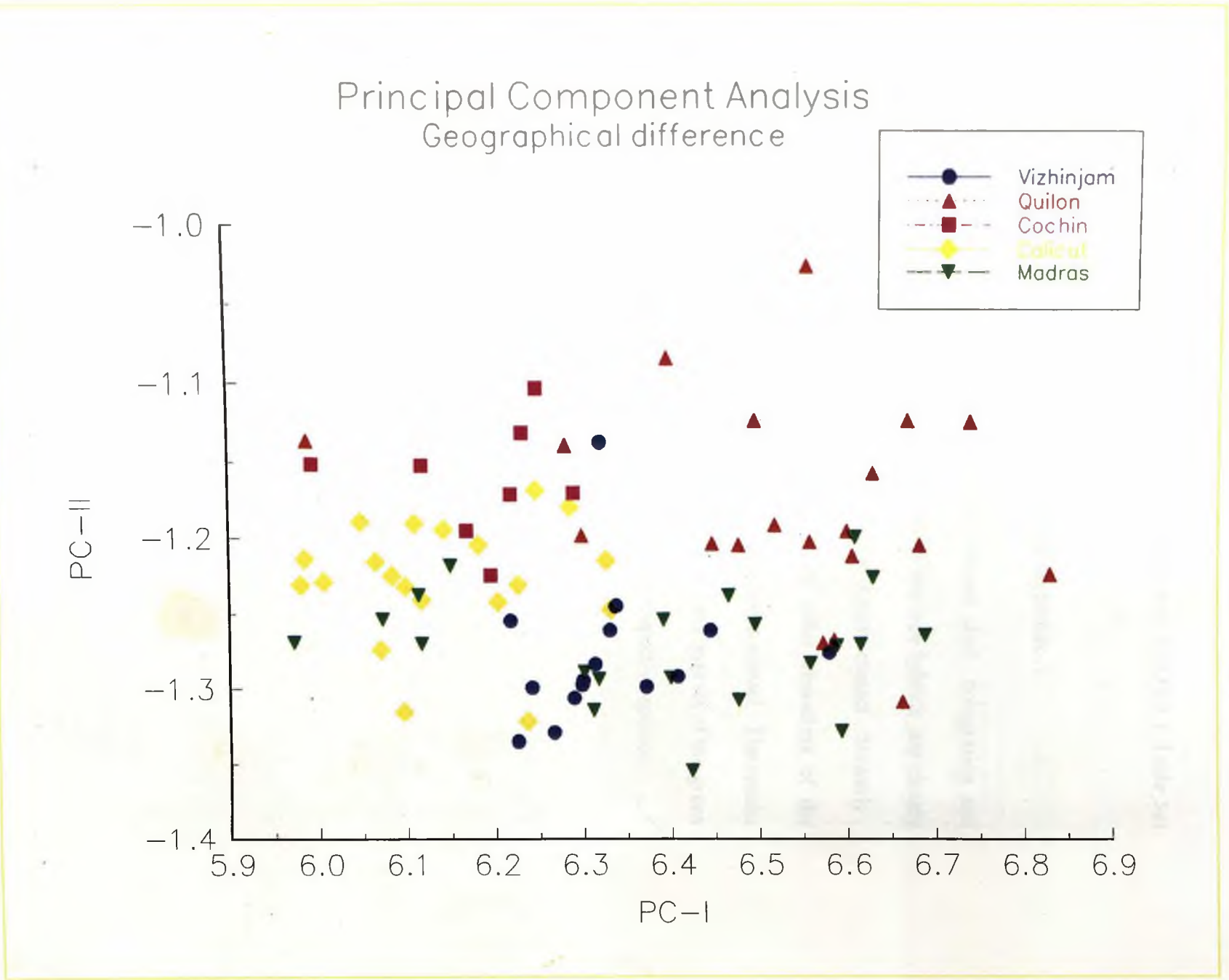
4.7.1. Discriminent analysis :

From the result of the discriminent analysis, it is concluded that both green and brown mussels are morphologically two distinct species (Table 6). According to the result of discriminent analysis,since $P(G_1/D)<(G_2/D)$(Table 7b) the suspected brown type hybrid,whose shell shape is morphologically similar to brown but having external shell colouration like the green mussel, is actually having the same morphomertics of the brown mussel. Similary, it is interesting to note that since $P(G_1/D)$ P (G_2/D) (Table 7a) the suspected green type hybrid whose shell shape is similar to green mussel but having brown colour on top with green posterior edge is actually having the same morphometrics of the green mussel.Therefore, the suspected brown and green type hybrids are not hybrids.Since their morphometrics are not different from the respective species these green and brown types are not varieties of the green or brown mussels.

4.7.2.Principal component analysis:

To examine the regional differences among the green mussel populations PC II were plotted against PC-I scores (Fig 5). From the plot, only Cochin samples form separate group from Vizhinjam, Quilon and Madras samples.All others do not form any clear grouping. Thus one may infer that Cochin green mussel is morphometrically different from that Vizhinjam, Quilon, and Madras.

Fig. 5.


Principal Component Analysis
Geographical difference
Vizhinjam
Quilon
Cochin
Calicut
Madras
−1.0
−1.1
−1.2
−1.3
−1.4
PC−II
5.9
6.0
6.1
6.2
6.3
6.4
6.5
6.6
6.7
6.8
6.9
PC−I

Cochin is not that different from Calicut stock .One way ANOVA (Table 5c) have shown significant difference between centres.

4.7.3. Morphometrics of two suspected hybrids :

All the morphological characters except external shell colouration and number of hinge teeth (Table 3) of the suspected brown type hybrids are closely comparable with the morphological characters of the brown mussel.. Similarly , except the external shell colouration (Table.3) all other characters of the suspected green type hybrids are similar to that of the green mussel. The results shows that the suspected brown and geen type hybrids are not hybrids of the green and brown mussels but are different colour morphs of the respective species.

Table 3. Diagnostic characters of two suspected hybrids :-

Diagnostic Characters		Brown type hybrids	Green type hybrids
1	Shape of the anterior end	Pointed and straight	Pointed, beak-like down turned
2	Size of hinge plate	Thick, narrow, terminal	Thick, broad, extends slightly to the ventral border
3	Number and size of hinge teeth	One teeth at the centre of the left valve and a corresponding depression on the right valve, a small teeth is seen on the lefthand side of the right valve and a corresponding depression on the left valve	Two small on the left valve and one on the right valve.
4	Dorsal ligamental margin	Straight	Moderately curved
5	Mid dorsal shell margin	A distinct dorsal angle or hump present	Moderately arcuate
6	Ventral shell margin	Almost straight	Highly concave
7	External shell colour	Light green with yellowish tinge on top ventral position	Yellowish-brown but the posterior edge of the external shell margin is dark green
8	Mantle margin colour	Yellowish-brown	Yellowish- green
9	Ventral Mantle margin	Inner fold of the posterior mantle margin thick, not extensible, smooth, with thick branching tentacles or papillae	Inner fold of the posterior ventral margin thin, extensible, smooth, tentacle or papillae absent.
10	Posterior byssal retractors	Two, Short, thick bundle anterior bundle arises from the posterior and diverges in the form of a 'V'	Two , short, thick bundles; anterior bundle arises form the posterior and diverges in the form of a 'V'.
11	Shell shape	Similar to that of the brown mussel	Similar to that of the green mussel

Table 4. Morphometric measurements of the mussels collected from different regions.

*CENTRE	**SPECIES	DVL (in mm)	WIDTH (in mm)	THICKNESS (in mm)
1	1	84.05	38.75	24.05
1	1	84.40	45.65	27.20
1	1	78.0	42.15	25.20
1	1	73.30	37.95	20.65
1	1	73.0	35.15	21.85
1	1	80.30	39.40	23.35
1	1	74.90	38.65	21.30
1	1	77.30	37.65	22.30
1	1	75.30	38.55	22.35
1	1	73.85	39.35	23.40
1	1	80.35	31.10	25.55
1	1	76.80	37.85	22.00
1	1	77.70	37.60	22.75
1	1	77.15	37.50	23.80
1	1	72.80	31.65	21.45
1	2	76.95	39.50	25.90
1	2	74.10	38.00	24.00
1	2	68.55	37.55	26.00
1	2	69.55	36.90	23.25
1	2	62.00	34.40	22.25
1	2	66.50	38.80	24.00
1	2	63.30	34.50	21.80

Contd.....

1	2	61.10	37.80	22.30
1	2	63.20	36.60	22.25
1	2	60.40	36.40	23.20
1	2	68.40	35.00	22.50
1	2	67.75	35.50	23.60
1	2	70.00	37.10	25.00
1	2	65.40	36.50	21.60
1	2	65.10	35.80	23.80
1	2	66.75	34.20	20.60
1	2	63.00	35.10	22.10
1	2	68.80	35.60	23.40
1	2	71.90	36.00	25.00
1	2	65.50	36.10	24.10
1	2	53.60	24.20	17.90
1	2	55.00	28.00	19.00
1	2	56.10	29.80	18.90
1	2	56.40	30.30	17.40
1	2	51.80	25.30	18.25
1	2	59.20	25.30	16.00
1	2	51.80	26.55	18.55
1	2	49.40	27.15	17.15
1	2	51.90	26.55	18.15
1	2	51.80	26.70	17.25
1	3	72.80	42.35	25.10
1	3	67.65	37.30	22.20
1	3	67.45	35.50	23.90
1	3	65.55	38.45	22.65
1	3	63.40	34.35	22.50
1	3	64.65	35.30	22.0

Contd.....

1	3	59.10	30.20	21.45
2	1	90.25	40.90	32.35
2	1	74.15	40.20	32.30
2	1	73.20	36.30	24.05
2	1	57.30	31.35	20.60
2	1	80.30	40.30	26.85
2	1	76.30	32.40	24.85
2	1	72.25	36.75	27.90
2	1	85.90	41.05	28.35
2	1	82.65	37.10	28.90
2	1	82.00	38.20	26.35
2	1	102.00	47.25	33..30
2	1	95.90	41.65	33.85
2	1	92.65	39.45	30.75
2	1	90.80	44.45	30.67
2	1	89.65	41.80	29.05
2	1	89.10	41015	29.30
2	1	92.60	41.65	27.50
2	1	98.95	44.00	28.00
2	1	96.05	39.70	27.25
3	1	85.55	39.10	27.85
3	1	74.35	32.80	22.05
3	1	72.20	32.05	25.00
3	1	72.05	32.05	22.15
3	1	74.45	34.50	24.42
3	1	73.88	32.24	23.31
3	1	72.27	32.20	22.23
3	1	67.70	31.00	22.15

Contd.....

3	1	62.80	29.25	20.45
3	4	104.10	43.50	30.05
3	4	91.50	41.25	30.00
3	4	87.80	38.20	28.90
3	4	86.25	33.80	28.00
3	1	80.90	36.50	24.70
3	4	66.80	33.80	23.25
3	4	96.05	38.95	27.00
3	4	75.80	31.85	26.45
3	4	62.75	31.90	21.65
3	4	58.70	33.30	21.25
4	1	80.80	34.50	21.05
4	1	77.30	31.70	23.80
4	1	76.90	33.00	22.40
4	1	74.50	32.00	19.30
4	1	73.10	31.10	20.70
4	1	67.75	29.50	19.45
4	1	68.10	28.60	19.10
4	1	68.50	30.70	20.40
4	1	81.50	35.35	23.70
4	1	72.15	31.10	21.80
4	1	71.90	30.10	20.50
4	1	70.60	28.60	20.60
4	1	74.70	34.50	24.20
4	1	70.80	31.50	19.60
4	1	80.20	34.70	24.20
4	1	75.20	31.40	22.20
4	1	72.10	30.70	20.60
4	1	79.50	31.50	21.90

Contd.....

4	1	69.70	30.90	21.40
4	1	66.90	28.80	19.40
4	1	99.40	40.25	28
4	1	96.00	38.80	29.40
4	1	100	41.20	26.40
4	1	81.60	37.90	24.50
4	1	87.70	36.90	24.50
4	1	80.30	36.10	22.50
5	1	73.60	31.40	21.50
5	1	72.10	31.35	20.70
5	1	67.00	31.35	20.70
5	1	101.20	42.50	29.550
5	1	93.35	41.75	29.20
5	1	89.50	38.80	26.15
5	1	93.70	40.60	26.70
5	1	95.35	40.75	27.50
5	1	86.90	38.00	26.01
5	1	90.00	39.60	24.85
5	1	89.40	39.30	23.15
5	1	81.15	37.00	22.20
5	1	82.50	35.90	22.65
5	1	72.25	32.30	20.25
5	1	66.75	29.95	18.45

*Centre Code

Vizhinjam-1

Quilon - 2

Cochin- 3

Calicut - 4

Madras - 5

**Species Code

Green - 1

Brown - 2

Suspected 'brown type' hybrids - 3

Suspected ' green type' hybrids - 4

Table5. Results of principal component analysis :-

Measurements DVL, width (W), Thickness (T)

Species Green mussel

Code No	Centres :
1	Vizhinjam (15)
2	Quilon (20).
3	Cochin (8)
4	Calicut (20).
5	Madras (21).

(a)

PC	*Eigen value	Percentage	Cum.percentage
1	3.7002	86.33	86.33
2	0.3384	7.90	94.22
3	0.2476	5.78	100.00

(b) Eigen vectors :

IM M:

0.527305 - 0.456946 0.716345 :

0.548335 - 0.461014 - 0.697707 :

0.649059 0.700702 0.007465 :

HM M<

* Eigen value is the ratio of the between groups to within groups sums of squares. Large eigen values are associated with good functions (Morusis,1993)

(c) Analysis of variance (anova) with pc scores

	Source	df	SS	MS	F	Probability
I with PC-I	Between centres	4	1.7571	0.4393	17.8590	0.00001
-	Within centres	79	1.9431	0.0246	-	
	Total	83	3.7002			
II with PC-II	Between centres	4	0.1563	0.0391	16.9278	0
	Within centres	79	0.1823	0.0023		
	Total	83	0.3386			
III with PC-III	Between centres	4	0.1130	0.0283	16.5878	0
	Within centres	79	0.1346	0.0017		
	Total	83	0.2476			

PC I → DVL

PC II → Width

PC III → Thickness

Table 6. Results of discriminent analysis for grouping of green and brown mussel

(a) Group means and standard deviations

Species	Number	DVL	W	T
1	63	77.90714	35.82603	24.24254
		(8.78993)	(4.71383)	(3.76253)
2	30	62.50833	33.22667	21.50667
		(7.41511)	(4.76521)	(2.91943)
Total	93	72.93978	34.98753	23.36
		(11.03541)	(4.86053)	(3.72564)

(b) Pooled within - groups correlation matrix

	DVL	W	T
DVL	1.0		
W	0.80953	1.0	
T	0.81121	0.80222	1.0

(c) Wilks Lambda (U-Statistic) and univariate F-ratio with 1 and 91 df

	Wilks Lambda	F	Significance
DVL	0.56988	68.68	.0000
W	0.93682	6.137	.0151
T	0.88088	12.31	0.0007

(d) Canonical Discriminant Functions

Function	Eigen Value	% of variance	Cum %	Cononical correlution
1*	1.4056	100	100	0.7644

After wilks

Function	Wilks lambda	Chi-squre	df	Significance
0	0.4157	78.563	3	0

* Marks the 1 canonical discriminant functions remaining in the analysis

(e) Unstandardised canonical Discriminant function remaining in the analysis

	FUNC 1
DVL	0.2176293
W	- 0.1893082
T	- 0.1280625
Constant	- 6.258869

Discriminant Function (DF)

= 0.2176293 DVL - 0.1893082 W - 0.1280625 T - 6.258869

(f) Canonical DF evaluated at Group means (Group centroids)

Group	Fun1
1	0.80929
2	- 1.69950

Case No	Actual Group	Highest Group	Probability		2nd Highest		Discrim Score
			P (D/G)	P(G/D)	Group	P(G/D)	
1	1	1	.4191	.9944	2	.0056	1.6173
2	1	1	.4091	.7458	2	.2542	-.0162
3	1**	2	.2266	.5283	1	.4717	-.4903
4	1	1	.3448	.6850	2	.3150	-.1354
5	1	1	.5264	.8260	2	.1740	.1757
6	1	1	.9149	.9468	2	.0532	.7025
7	1	1	.4167	.7520	2	.2480	-.0029
8	1	1	.8191	.9291	2	.0709	.5806
9	1	1	.4005	.7384	2	.2616	-.0314
10	1**	2	.2861	.6156	1	.3844	-.6329
11	1	1	.2848	.9971	2	.0029	1.8789
12	1	1	.7362	.9090	2	.0910	.4724
13	1	1	.8495	.9353	2	.0647	.6195
14	1	1	.6708	.8890	2	.1110	.3843
15	1	1	.2717	.5962	2	.4038	-.2898
16	2**	1	.2644	.5859	2	.4141	-.3068
17	2**	1	.2266	.5284	2	.4716	-.3997
18	2	2	.9370	.9659	1	.0341	-1.7785
19	2	2	.5393	.8330	1	.1670	-1.0857
20	2	2	.6687	.9855	1	.0145	-2.1274
21	2	2	.6131	.9881	1	.0119	-2.2052
22	2	2	.9153	.9681	1	.0319	-1.8058
23	2	2	.2027	.9982	1	.0018	-2.9734
24	2	2	.5597	.9901	1	.0099	-2.2828
25	2	2	.2299	.9979	1	.0021	-2.9002

Contd...

26	2	2	.4126	.7487	1	.2513	-.8802
27	2	2	.6583	.8827	1	.1153	-1.2572
28	2	2	.6529	.9590	1	.1173	-1.2497
29	2	2	.9982	.9757	1	.0410	-1.7018
30	2	2	.8283	.7315	1	.0243	-1.9163
31	2	2	.3926	.9813	1	.2685	-.8445
32	2	2	.7462	.9813	1	.0187	-2.0231
33	2	2	.4981	.8096	1	.1904	-1.0220
34	2	2	.2839	.6127	1	.3873	-.6280
35	2	2	.8220	.9761	1	.0239	-1.9245
36	2	2	.8165	.9286	1	.0714	-1.4675
37	2	2	.7463	.9813	1	.0187	-2.0231
38	2	2	.6802	.9849	1	.0151	-2.1116
39	2	2	.8031	.9775	1	.0225	-1.9489
40	2	2	.6797	.9850	1	.0150	-2.1123
41	2	2	.3063	.6412	2	.3588	-.2137
42	2**	1	.4915	.9924	1	.0076	-2.3874
43	2	2	.2524	.9976	1	.0024	-2.8440
44	2	2	.5386	.9909	1	.0091	-2.3144
45	2	2	.5825	.9893	1	.0107	-2.2493
46	2	2	.9366	.9660	2	.0340	.8888
47	1	1	.5043	.8133	2	.1867	.1416
48	1	1	.7704	.9179	2	.0821	.5174
49	1	1	.6002	.8620	2	.1380	.2852
50	1	1	.9377	.9503	2	.0497	.7312
51	1	1	.5900	.8576	2	.1424	.2705
52	1	1	.2984	.6314	2	.3686	-.2305
53	1	1	.3413	.6813	1	.3187	-.7479
54	1	1	.1972	.9983	2	.0017	2.0987

Contd.....

55	1	1	.4804	.9927	2	.0073	1.5149
56	1	1	.5811	.9893	2	.0107	1.3611
57	1	1	.5381	.9909	2	.0091	1.4250
58	1	1	.7625	.9803	2	.0197	1.1115
59	1	1	.6898	.8953	2	.1047	.4101
60	1	1	.9141	.9467	2	.0533	.7015
61	1	1	.5587	.8429	2	.1571	.2245
62	1	1	.3464	.9960	2	.0040	1.7508
63	1	1	.9637	.9540	2	.0460	.7638
64	1	1	.7980	.9779	2	.0221	1.0652
65	1	1	.8071	.9772	2	.0228	1.0535
66	1	1	.6589	.8849	2	.1151	.3678
67	1	1	.8940	.9434	2	.0566	.6761
68	1	1	.4730	.9929	2	.0071	1.5269
69	1	1	.6098	.9882	2	.0118	1.3196
70	1	1	.8626	.9729	2	.0271	.9824
71	1	1	.1428	.9989	2	.0011	2.2749
72	1	1	.6244	.8720	2	.1280	.3197
73	1	1	.6561	.8839	2	.1161	.3640
74	1	1	.4919	.9924	2	.0076	1.4966
75	1**	2	.8660	.9726	1	.0274	-1.8683
76	1	1	.2759	.6020	2	.3980	-.2802
77	1**	2	.5079	.9919	1	.0081	-2.3616
78	1	1	.5092	.8162	2	.1838	.1492
79	1	1	.8251	.9759	2	.0241	1.0303
80	1**	2	.5259	.8257	1	.1743	-1.0652
81	1	1	.8223	.9761	2	.0239	1.0338
82	1	1	.8457	9743	2	.0257	1.0039
83	1	1	.8639	.9728	2	.0272	.9807

Contd.....

84	1	1	.0548	.9997	2	.0003	2.7300
85	1	1	.1134	9992	2	.0008	2.3922
86	1	1	.0912	.9994	2	.0006	2.4984
87	1	1	.7262	.9825	2	.0175	1.1594
88	1	1	.4185	.9944	2	.0056	1.6183
89	1	1	.4352	.9940	2	.0060	1.5896
90	1	1	.0934	.9994	2	.0006	2.4872
91	1	1	.0107	.9999	2	.0001	3.3602
92	1	1	.0047	1.0000	2	.0000	3.6392
93	1	1	.5609	.9901	2	.0099	1.3908

** mis identified

(g) Classification results :-

Actual group	No. of cases	Predicted group membership			
		1		2	
1 (Green)	63	57	90.5%	6	9.5%
2 (Brown)	30	3	10.0%	27	90%

Percent "grouped" cases correctly classified : 90.32%

(h) Summaries of Discore 1 Discriminent Score

By levels of Species

Variable Value level Mean SD

Cases

For entire population 1.4516E-15 1.5425511 93

Species 1 (Green) 0.8092865 1.0946477 63

Species 2 (Brown) -1.6995016 0.7590437 30

(i) ANOVA

Source	SS	df	MS	F	Sig
Between groups	127.9107	1	127.9107	127.9107	0.0000
Within groups	91.0	91	1.0		

Eta 0.7644 Eta squared 0.5843

Table 7. Result of discriminent analysis for suspected individuals collected from Cochin and Vizhinjam :-

(a) Cochin (Suspected 'green type' hybrids)

Case Number	Discrim score	Z_1	P (D/G_1)	$P(G_1/D)$	Z_2	$P(D/G_2)$	$P(G_2/D)$	Remark
1	4 18509	3.084	0.0035	1.00	7.75	0	0	$P(G_1/D)>$ $P(G_2/D)$
2.	2.00337	1.09	0.2203	1.00	4.88	0	0	"
3.	1.91640	1.0114	0.2396	1.00	7.76	0	0	"
4.	2.52729	1.57	0.1163	1.00	5.57	0	0	"
5.	1.27444	0.42	0.3653	0.9986	3.92	0.002	0.0014	"
6	-1.09730	-1.741	0.0878	0.5624	0.793	0.292	0.4376	"
7.	3.813183	2.744	0.0093	1.00	7.26	0	0	"
8.	0.82071	0.01	0.3989	0.9990	3.32	0.0016	0.001	"
9.	-1.41411	-2.03	0.0508	0.3946	0.376	0.3847	0.6054	$P(G_1/D)<$ $P(G_2/D)$
10	0.08394	-0.66	0.3209	0.9800	2.35	0.0252	0.02	$P(G_1/D)>$ $P(G_2/D)$

b. Vizhinjam (Suspected 'brown type' hybrids)

Case Number	Discrim score	Z_1	P (D/G_1)	P(G_1/D)	Z_2	P(D/G_2)	P(G_2/D)	Remark
1.	-o.0135	-075164	0.3011	0.2329	2.221218	0.0519	0.7661	P(G_2/D)>P(G_1/D)
2.	0.0611	-0.68349	0.3166	0.2469	2.319499	0.0508	0.7531	"
3.	-1.1068	-1.75041	0.0863	0.0152	0.780853	0.2943	0.9848	"
4.	-0.7949	-1.46548	0.1374	0.3549	1.191764	0.1965	0.6451	"
5.	-1.4869	-2.0976	0.0449	0.0061	0.280091	0.3836	0.99	"
6.	-0.7758	-1.44803	0.1415	0.0373	1.216928	0.1919	0.9627	"
7.	-2.2642	-2.80774	0.0079	0.0014	-0.74396	0.3034	0.9986	"

Table 8. Staining intensity and thickness of esterase bands in different tissues of Green, Brown and suspected 'brown type' hybrid mussels.

a)

Mantle						Gill					
Green		Brown type hybrid		Brown		Green		Brown type hybrid		Brown	
Thickness of bands	Staining Intensity	Thickness of bands	Staining Intensity	Thickness of bands	Staining Intensity	Thickness of bands	Staining Intensity	Thickness of bands	Staining Intensity	Thickness of bands	Staining Intensity
0.6-1.0	1x	0.5-0.7	1x	0.5-0.7	1x	0.3	1x	0.5-0.9	1x	0.5-0.9	1x
2.0-2.1	2x	1.1-1.2	1x	1.1-1.2	1x	0.5	1x	2.3-2.6	2x	2.3-2.6	2x
2.8-2.9	3x	2.5-2.7	1x	2.5-2.7	1x	1-1.2	2x	4.8-4.9	2x	4.8-4.9	2x
3.9-4.2	3x	4.2-4.5	3x	4.2-4.5	3x	1.6	2x	5.3-5.4	2x	-	-
-	-	-	-	-	-	3-3.2	3x	5.8-5.9	2x	-	-
-	-	-	-	-	-	4.2	1x	-	-	-	-
-	-	-	-	-	-	4.7	1x	-	-	-	

b)

Adductor Muscle						Gill					
Green		Brown type hybrid		Brown		Green		Brown type hybrid		Brown	
Thickness of bands	Staining Intensity	Thickness of bands	Staining Intensity	Thickness of bands	Staining Intensity	Thickness of bands	Staining Intensity	Thickness of bands	Staining Intensity	Thickness of bands	Staining Intensity
1.3-1.5	1x	0.5-0.7	1x	0.5-0.7	1x	0.6-1.0	2x	0.6-1.0	2x	0.6-1.0	2x
1.9-2.0	2x	1.5-1.6	1x	1.5-1.6	1x	2-2.5	2x	1.9-2.4	2x	2.0-2.5	2x
2.2-2.3	1x	2.5-2.6	1x	2.5-2.6	1x	2.9-3.0	2x	4.3-4.4	2x	-	-
2.4-2.5	1x	4.7-4.8	3x	4.7-4.9	3x	4-4.1	2x	4.7-4.8	1x	4.7-4.8	1x
3.3-3.5	3x	-	-	-	-	4.4-4.5	2x	-	-	-	-
4.4-4.5	3x	-	-	-	-	-		-	-	-	-

Table 9. Staining intensty and thickness of MDH bands in adductor muscle of green, brown and suspected 'brown type' hybrid mussels

Adductor muscle					
Green		Brown type hybrid		Brown	
Thickness of bands	Staining Intensity	Thickness of bands	Staining Intensity	Thickness of bands	Staining Intensity
2.0-2.5	3x	3.0-3.1	2x	3.0-3.1	2x
2.9-3.0	2x	3.3-4.0	2x	3.3-4.0	2x
3.3-3.5	2x	-		-	-

Table 10. Staining intensity and thickness of general protein bands in different tissues of green, brown and suspected 'brown type' hybrid mussels

a)

Adductor Muscle						Mantle					
Green		Brown type		Brown		Green		Brown type		Brown	
Thickness	Staining	Thickness	Staining	Thickness	Staining	Thickness	Staining	Thickness	Staining	Thickness	Staining
0.3-0.4	1x	0-0.1	2x	0.0-0.1	2x	1.9-2.0	2x	2-2.2	2x	2-2.2	2x
0.6-0.7	1x	0.4-0.5	2x	0.4-0.5	2x	3.0-3.1	2x	2.4-2.5	2x	2.4-2.5	2x
1.9-2.1	4x	0.7-0.8	2x	0.7-0.8	2x	3.5-3.6	2x	3.5-3.6	2x	3.5-3.6	2x
2.3-2.4	1x	1-1.1	2x	1-1.1	2x	3.8-3.9	2x	3.8-3.9	2x	3.8-3.9	2x
2.9-3	1x	1.3-1.4	2x	1.3-1.4	2x	4.1-4.3	4x	4.6-4.8	4x	4.6-4.8	2x
3.2-3.3	2x	2.3-2.5	4x	2.3-2.5	4x	4.7-4.9	1x	5.4-5.6	2x	5.4-5.6	4x
3.9-4.1	4x	2.7-2.8	2x	2.7-2.8	2x	5.4-5.5	1x	6.0-6.2	2x	6.0-6.2	2x
4.2-4.3	2x	3.0-3.1	2x	3.0-3.1	2x	5.8-6.0	3x	6.5-6.6	2x	6.5-6.6	2x
4.5-4.7	2x	3.3-3.5	2x	3.3-3.5	2x	6.7-6.9	1x	-	-	6.8-69	1x
6-6.2	1x	3.7-3.8	2x	3.7-3.8	2x	7.6-7.7	2x	7.6-7.7	2x	7.6-7.7	2x
6.5-6.7	2x	4.1-4.2	2x	4.1-4.2	2x	-	-	-	-	-	-
7-7.2	2x	4.3-4.4	2x	4.3-4.4	2x	-	-	-	-	-	-
7.4-7.5	2x	4.4-4.6	3x	4.4-4.6	3x	-	-	-	-	-	-
-	-	4.7-4.9	2x	4.7-4.9	2x	-	-	-	-	-	-
-	-	5.3-5.5	1x	5.3-5.5	1x	-	-	-	-	-	-
-	-	6.0-6.1	1x	6.0-6.1	1x	-	-	-	-	-	-
-	-	6.5-6.7	2x	6.5-6.7	2x	-	-	-	-	-	-
-	-	7.1-7.2	1x	7.1-7.2	1x	-	-	-	-	-	-
-	-	7.3-7.4	1x	7.3-7.4	1x	-	-	-	-	-	-
-	-	7.6	1x	7.6	1x	-	-	-	-	-	-

b)

Foot						Gill					
Green		Brown type		Brown		Green		Brown type		Brown	
Thickness of bands	Staining Intensity	Thickness of bands	Staining Intensity	Thickness of bands	Staining Intensity	Thickness of bands	Staining Intensity	Thickness of bands	Staining Intensity	Thickness of bands	Staining Intensity
0.5	2x	0.5	2x	0.5	2x	0.6	2x	0.6	2x	0.6	1x
1.1-1.3	2x	1.4-1.5	2x	3.1-3.2	3x	0.7	2x	1.8-1.9	2x	1.8-1.9	1x
1.6-1.7	3x	1.8-1.9	2x	3.7-3.8	2x	1.8-1.9	2x	2.5-2.7	3x	2.5-2.7	4x
2.5-2.7	4x	3.0-3.2	3x	4.4-4.5	1x	2-2.2	3x	3.8-3.9	1x	3.8-3.9	2x
3.0-3.2	3x	3.5-3.6	2x	5.7-6.0	3x	3.1-3.3	1x	5.2-5.4	3x	5.2-5.4	3x
4.2-4.3	2x	4.3-4.4	1x	6.1-6.3	2x	3.7-3.8	1x	5.8-5.9	1x	5.8-5.9	1x
5.0-5.3	4x	5.7-6.0	4x	7.1-7.3	2x	4.5-4.7	3x	6.5-6.7	2x	6.5-6.7	2x
5.6-5.9	2x	6.1-6.3	2x	7.4-7.5	1x	5.8-5.9	1x	7.1-7.2	1x	6.7-6.9	2x
6.1-6.3	2x	7.1-7.3	2x	7.6-7.8	1x	6.5-6.7	2x	7.3-7.4	1x	7.1-7.2	1x
6.8-6.9	1x	7.6-7.8	1x			7.3-7.4	1x			7.3-7.4	1x
7.1-7.2	1x	-	-	-	-	-	-	-	-	-	-
7.6	1x	-	-	-	-	-	-	-	-	-	-
7.8	1x	-	-	-	-	-	-	-	-	-	-

Table 11. Staining intensity and thckness of general protein bands in different tissues of Green, Brown and suspected 'Green type' hybrid mussels

Adductor Muscle						Mantle					
Green		Green type		Brown		Green		Green type		Brown	
Thickness of bands	Staining Intensity	Thickness of bands	Staining Intensity	Thickness of bands	Staining Intensity	Thickness of bands	Staining Intensity	Thickness of bands	Staining Intensity	Brown	Staining Intensity
0.6	2x	0.6	2x	0.0-0.1	1x	0.4	2x	0.4	2x	4.0-4.1	4x
1.0	2x	1.0	2x	0.4-0.5	2x	0.5	2x	0.5	2x	4.3-4.4	4x
2.5-2.6	2x	2.5-2.6	2x	1.0	2x	2.2	2x	2.2	2x	5-5.2	3x
3.7-3.8	1x	3.7-3.8	1x	2.0-2.2	4x	2.4-2.5	1x	2.4-2.5	1x	6.5-6.6	2x
4.3-4.5	4x	4.3-4.5	4x	2.5-2.6	2x	2.7-2.8	4x	2.7-2.8	4x	6.7-6.8	2x
5-5.1	1x	5-5.1	1x	3.7-3.8	1x	4.0-4.1	4x	4.0-4.1	4x	7.3-7.4	2x
6.5-6.6	2x	6.5-6.6	2x	4.0-4.1	4x	4.3-4.4	4x	4.3-4.4	4x		
6.7-6.8	2x	6.7-6.8	2x	4.3-4.5	2x	4.5	3x	4.5	3x		
7.4-7.5	2x	7.4-7.5	2x	5.0-5.1	1x	4.6-4.7	2x	4.6-4.7	2x		
7.7-7.8	2x	7.7-7.8	2x	6.5-6.6	1x	5.0-5.2	1x	5.0-5.2	1x		
	2x			7.4-7.5	2x	0.5-6.6	3x	0.5-6.6	3x		
				7.8	2x	7.3-7.4	1x	7.3-7.4	1x		
						7.6	2x	7.6	2x		

Table 12. Staining intensity and thickness of general proten bands in mantle tissues of Green mussels from different regions

Calicut		Madras		Cochn		Quilon		Vizhinjam	
Thickness of bands	Staining Intensity	Thickness of bands	Staining Intensity	Thickness of bands	Staining Intensity	Thickness of bands	Staining Intensity	Thickness of bands	Staining Intensity
0.5	1x	0.5	2x	0.5	2x	0.5	1x	1.0	2x
1.0	1x	1.0	2x	1.0	2x	0.6	1x	1.6	1x
1.3	2x	1.6-1.7	2x	1.6-1.7	1x	1.0	1x	3-3.1	2x
1.6-1.7	1x	2.5-2.6	2x	2.5-2.6	2x	2.5-2.6	1x	3.2-3.3	2x
2.5-2.6	2x	2.9-3	3x	3.0-3.1	2x	2.8-2.9	2x	3.8-3.9	2x
3-3.1	3x	3.3-3.4	2x	3.2-3.3	2x	3.1-3.2	2x	4.1-4.2	2x
3.4-3.6	2x	3.8-3.9	2x	3.7-3.8	1x	3.8-3.9	2x	4.8-4.9	3x
3.8-3.9	2x	4.0	2x	4.0-4.1	1x	4.0-4.1	1x	5.1-5.2	4x
4.2-4.3	1x	4.8-4.9	2x	4.8-4.9	4x	4.8-4.9	4x	5.9-6.0	1x
4.8-4.9	4x	5.1-5.2	4x	5.1-5.2	2x	5.1-5.2	4x	6.4-6.5	1x
5.1-5.2	4x	5.9-6.0	1x	6.0-6.2	1x	6.0-6.2	1x	7.0-7.1	2x
5.9-6.1	1x	6.7-6.8	1x	6.8-7.0	1x	6.5-6.7	1x	7.5-7.6	1x
6.7-6.8	1x	7.5-7.6	1x	7.2-7.3	1x	-	-	7.8-7.9	1x
7.4-7.5	1x	7.8-7.9	1x	7.7-7.8	1x	-	-	-	-
7.9	1x	-	-			-	-	-	-

5. Discussion

The mussels within the Mytilidae have confusing morphological features. Accurate identifications of these mussels is a challenging job to the taxonomists. As a result, many species under the Mytilidae have been redescribed or wrongly classified. The *Mytilus* and *Perna* are the major genus reported under the Mytilidae. The taxonomic interrelationships of these two genus and their more than 470 species have also been greatly confused (Siddall, 1980). The major reason for such confusion, appears to be the consequences of the environmentally influenced, non-genetic morphological/anatomical, often overlapping characteristics used for taxonomic purposes (Seed 1968, Gosling 1992b).

The green and brown mussels of India was described, until recently, as *Mytilus* species (Annandale 1916, Hornell, 1921, Gravely, 1941, Menon *et al.*, 1966). However, detailed investigation and analysis of its morphological/anatomical features enabled Kuriakose and Nair (1976) to report that the Indian green and brown mussels actually belong to the genus *Perna* and not to the genus *Mytilus*. Thus the green mussel was placed under the species name *P. viridis* while brown mussel was described as new species under the name *P. indica*. However, the question is that whether these morphologically different species of the green and brown mussels are genetically different species ? It is very well known that genetically controlled proteins/enzymes are present in tissues of organisms are potential genetic markers for accurate identification of the species (Ferguson 1980, Murphy, *et al.*, 1990). Thus the taxonomic problems in fishes

(Smith *et al* , 1979, Smith and Robertson, 1981), molluscs (Richardson *et al.*, 1982), prawns (Boulton and Knott, 1984) have been solved by detecting species specific tissue proteins/enzyme separated by gel electrophoresis. Species of *Mytilus* have been easily identified using electrophoretic profiles of prtoeins (Koehn *et al.*, 1984, Vainola and Hvilsom, 1991; Suchanek *et al.*, 1997). Even the species source of fish fillets can be detected by comparative study of tissue proteins (Shaklee and Keenan, 1986). Since hybrid protein/enzyme molecules can be directly detected by the gel electrophoresis, the problem of suspected hybrid species have also been easily solved (Shearer and Mulley, 1978; Rowland, 1984; Pullan and Smith, 1987). The inter species genetic differences revealed by the electrophoretic profiles of the tissue proteins in the Indian fishes (Manohar and Velankar, 1973, Menezes 1977, Mahobia, Chakroborty, 1990), prawns (Thomas, 1981, Philip Samuel, 1981) and crabs (Kannupandi and Paul Pandian, 1975) were reported earlier. But such reports with reference to Indian bivalves were not available. The present study and its results have filled up the gap.

Interestingly, the results of the present investigation have revealed that the electrophoretic profiles of the general proteins in the green mussel, *Perna viridis* and the brown mussel, *P.indica* are distinctly different and specific for each species. Thus the present studies have also proved that the green and brown mussels of the Indian coasts are two distinct species. The different protein profiles were revealed by the species specific tissue proteins present in the mantle, foot, adductor and gill. Among these four tissues, a single major protein band located at

5 to 5.3 cms in *P.viridis* and at 5.6 to 5.9 cms in *P. indica* was the strongest genetic marker that easily separated the two species (Fig.6). Besides, the electrophoretic profiles of the malate dehydrogenase and esterase enzymes obtained in these two species in the present study were also enabled to prove that the green and brown mussels are two distinct species. The MDH band lacated at 1.9 to 2.6 was characteristic of the green mussel while the band (s) present in the brown mussel was beyond 3 cm. Thus the electrophoretic profiles of general proteins and the enzymes, MDH and EST, clearly established that the green mussel *P.viridis* and *P. india* are two distinct species as reported originally by Kuriakose and Nair, (1976) on the basis of morphological /anatomical characteristics

The species specific protein profile in the brown and green mussels can be described more accurately in an alternate way by quantifying species specific gene loci in each species which actually controlled the production of the observed protein phenotypes (Ayala and Kiger, 1980). Though the total number of protein loci quantified in the gill, mantle, foot and adductor muscle was about 28 to 29 in the two species, majority of the loci each tissue was distinct for each species (Fig. 6) It means that the brown and green mussels of India are two distinct genetic species The enzyme gene loci were also species specific in the two species. It means that the morphological/ anatomical characteristics considered by Kuriakose and Nair (1976) were polygenic in nature and valid for the species under consideration (Table 1) It also means that wherever controversy on the

morphological/anatomical differentiation of two or more species arises, an electrophorectic profile of protein/enzymes should also be considered for settling the issue

It is also known that the populations within a species may get differentiated into races, varieties or subspecies etc. (Ayala and Kiger, 1980) A comparative study of specific protein/enzyme loci and estimate of the allele frequencies of the loci among populations has enabled investigators to detect such genetically differentiated stocks within fishes/shell fishes (Viard *et al* 1994)

In the present short term investigation, a preliminary attempt was also made to detect protein polymorphic loci in the samples of the green mussel from Vizhinjam, Quilon, Cochin, Calicut and Madras. Though the protein banding patterns at zones between 2 and 8 cms gel length starting from the point of protein extract application showed variable phenotypes in some individuals, the patterns were inconsistent and were not suitable for comparison of gene frequencies in the sampled populations

Another very valuable informations emerged during the present investigation were the results of testing the hypothesis that the mussel specimens showing colour of green mussel but having the shell shape of the brown mussel (brown type variety/hybrid) and those specimens showing colour of brown and green mussel and shell shape of the green mussel (green type variety/hybrid) are either hybrid types or of the varieties of the green and the brown mussels.

The electrophoretic profiles of the tissue proteins and the enzymes, malate dehydrogenase (MDH) and the esterase (EST) obtained in these assumed hybrids/varieties and the green/brown mussels revealed very interesting results. In true hybrids, the hybrid protein/enzyme patterns of the suspected parents are expected to occur. However, the suspected hybrid having shell shape of the brown mussel and shell colour of the green mussel showed almost identical protein and enzyme profiles of the brown mussel (*P.indica*). Similarly, the suspected hybrid having the shape of the green mussel with brown and green shell colour also showed identical protein/enzyme profiles of the green mussel *(P. viridis*). It means that these two apparent hybrids/varieties are only two different colour morphs of the corresponding species and not true hybrids or varieties as suspected. However, the shell colours of these suspected varieties/hybrids were different from the respective species and an explanation for the colour difference is required. It is reported that such colour morphs are not genetically inherited in the blue mussel *Mytilus edulis* (Gosling , 1992). It means that the different colour morphs in these two Indian *Perna* species are influenced by some non-genetic factors.

The discriminent analysis of the morphometrics of the green (*P.viridis*) and the brown (*P. indica*) mussels also produced very significantly different values. The results again confirm that the green and the brown mussels of India are two distinct morphological species. This is an additional information to that of Kuriakose and Nair (1976) as they did not apply the method of discriminant analysis for differentiating the two species. On the other hand, principal

component analysis of the morphometrics carried out for the first time among the population samples of the green mussel from Vizhinjam, Quilon, Cochin, Calicut and Madras has indicated that the shell morphometrics of the Cochin stock are different from that of all other regions. On the other hand, one way ANOVA analysis have shown that significant differences occur between centres. Morphologically different stocks of the mussel species have been reported by others (CMFRI, Bulletin 29)

As explained else where, the protein profiles of the samples of the green mussel did not show any significant variations to support the above significant morphological differences of the Calicut stock or between the centres. However, a detailed analysis of different enzyme loci of different green mussel populations should help to identify genetic stock heterogenesity within the species *P.viridis*.

Moreover, a detailed analysis of potential polymorphic enzyme loci, karyotypes and mitochondrial DNA types present in the *P. viridis*, and *P.indica* and the suspected hybrids is necessary to throw more light on the genetic structure of these species, their population and the genetic nature of the suspected hybrids. Meanwhile, the value of the present findings is clear. The green mussel, *P.viridis* and *P.indica* being genetically distinct species, these should be judicially exploited in order to conserve their distinct genetic resources.

6. Conclusions

1. The tissue protein and the enzyme (MDH and EST) profiles in the green (*Perna viridis*) and the brown (*P. indica*) mussels were found distinctly different. A striking genetic markar that easily differentiated the two species was the protein fraction (locus) designated as number 14 in the brown mussel and 15 in the green mussel (Fg.6). Large number of protein loci were distinctly different in the two species. The green and brown mussels of Indian coasts are genetically distinct species .

2. The protein profiles in the mantle, foot, adductor muscle and gill are tissue specific and species specific in the two species.

3. A comparative study of the protein profiles and the morphometrics in the suspected brown and green type hybrid mussels and the green/brown mussels revealed that the suspected hybrids are not, infact, hybrids but different colour morphs of the respective brown and/ the green mussels.

4. The principal component analysis produced significant values in the Calicut stock of the green mussel. The ANOVA of the morphometrics in the green mussel samples from Vizhinjam, Quilon, Cochin and Madras also produced significant values between the centres.

7. Summary

The dissertation contains the results of a short term investigation entitled " Electrophoretic profile of the general proteins in the green (*Perna viridis* Linnaeus) and the brown (*Perna indica* Kuriakose & Nair) mussels"

1. The aim of the investigation was of two fold. First, to study the electrophoretic profile of proteins in the green (*Perna viridis*) and brown (*P. indica*) mussels of India with a view to understanding that whether the controversial green and brown mussels are genetically distinct species. Second, to test the hypothesis that the mussel specimens having shell shape of the green or the brown mussels but shell colour of the other species or of both may be hybrid of the two species.

2. To achieve the above aim, samples of the green and brown mussels and specimens of suspected hybrids were collected..

3. Methods for extraction of general proteins from mantle, foot, adductor muscle and gill and their separation and detection by gel electrophoresis were standardized. A gel of 9% using Tris HCl/Tris-glycine buffer systems produced better separation and resolution of the protein fractions (Table 10)

4. The protein profiles in different tissues of the green mussel differed significantly from that of the brown mussel. The electrophoretic position of the many major or minor protein fractions of the green and brown mussel were different and species specific. Majority of the 28 to 29

protein fraction present in the gill, mantle, foot and adductor muscle tissue were quantified as species specific gene loci in the brown and green mussels. The most striking species specific protein fraction or the locus was revealed by the foot tissue. It was 14 in the brown and 15 in the green mussels (Fig 6) Similarly the electrophoretic profiles of the enzymes, MDH and esterases in the green and brown mussels were significantly different. Hence, the green and brown mussels of India are genetically different species.

5. The electrophoretic profiles of the proteins in different tissues of the green and the brown mussels were different and the pattern was tissue specific in each species on account of the migrating differences in majority of the protein fractions.

6. Discriminent analysis of morphometrics of the green and the brown mussels also showed that *P. viridis* and *P. indica* are morphologically two distinct species.

7. Discriminent analysis of the morphometrics of the green mussel (*P. viridis*) with the suspected green type hybrid and that of the brown mussel (*P. indica*) with that of the brown type hybrid revealed that the suspected hybrids are having identical morphometrics of the respective species. The suspected hybrids are not hybrids of the green and brown mussels but different colour morphs of the respective species.

8. The principal component analysis of the green mussel samples from Vizhinjam, Quilon, Cochin, Calicut and Madras showed that Calicut green mussel stock is different from the other regions. The ANOVA results indicated that the above regional populations of the green mussel have significantly different morphometrics.

9. Finally, it was suggested that a detailed analysis of the polymorphic enzyme loci, karyotypes and mitochondrial DNA types in the green and brown mussels and their suspected hybrids may be carried out in the future to throw more light on the topic.

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